# THE JOURNAL OF PHYSIOLOGY

EDITED FOR

## THE PHYSIOLOGICAL SOCIETY

BY


N. AMBACHE
M. DE B. DALY
G. GORDON
R. J. LINDEN
R. V. COXON
P. M. DANIEL
CATHERINE HEBB
P. A. MERTON
W. A. H. RUSHTON
G. S. BRINDLEY (*Secretary*)
D. J. C. CUNNINGHAM
S. P. DATTA
O. F. HUTTER
W. J. O'CONNOR
D. H. SMYTH
B. KATZ (*Chairman*)



August 1961

Volume 157, No. 3

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W. 1

American Branch: 32 East 57th Street, New York 22, N.Y.

*Price 30s. net*

*(U.S.A. $5.00)*

**Notice to Contributors.** Papers submitted for publication should be sent to:

THE EDITORS OF THE JOURNAL OF PHYSIOLOGY

*Physiology Department, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London, E.C.* 1

Contributors should follow the instructions in '**Suggestions to Authors**' with regard to the preparation of papers, and should study carefully the format of papers in recent numbers of the Journal. The latest edition of this pamphlet appeared in Vol. 150, p. 1. Offprints may be obtained from **The Cambridge University Press, 200 Euston Road, London, N.W. 1.** Price 2*s.* in paper cover, post free.

Papers submitted for publication should be accompanied by a declaration in the following terms: The attached paper entitled ..... has not been, and is not intended to be published anywhere except in the Journal of Physiology, and we/I agree that, if and when it is accepted by the Editors for publication, in consideration for such acceptance the entire copyright in this paper shall pass thereupon to the Physiological Society.

Requests for permission to reproduce any part of papers published in the Journal of Physiology should be addressed to the Secretary of the Editorial Board at the Physiological Laboratory, Cambridge. The Editorial Board will normally grant all requests from authors to reproduce material from their own papers. Permission will not be given to third parties except with the consent of the authors concerned, but if the Editorial Board receives no reply within a reasonable period of writing to the authors at their last address notified, consent will be assumed.

When a paper is submitted by several authors in collaboration their names will be printed in alphabetical order.

The date of receipt printed at the head of each paper is normally the date on which the typescript is first received by the Editors, but if a paper has to be extensively altered after submission the date may at the discretion of the Editors be changed to that on which an acceptable draft is received.

Authors are reminded that the acceptance of papers may be prevented by their being faulty in English composition. It is also essential that papers should be written as concisely as possible, without undue repetition of material in text, tables and figures. Details under the heading 'Methods' will appear in small print unless the author particularly wishes otherwise. Papers concerned with methods only are not acceptable, unless the methods involve new principles and are shown to be of proved value and interest to physiologists. Long theoretical discussions are in general unsuitable.

In an exceptional case the Editors will be prepared to accept a coloured illustration if they consider it to be necessary for conveying the scientific content of the paper. Half the cost of reproduction will be charged to the author.

Authors are requested to verify that the wording of references to unpublished work, and of acknowledgements of help or criticism, is approved by the persons concerned.

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of their typescripts and illustrations.

Proofs should be corrected and returned promptly. Unless otherwise directed, proofs are sent to the author whose name is first alphabetically, at the address from which the paper originates. If the author(s) wish the proofs to be sent to any other person, or to any other address, instructions should be given.

**Abstract.** Authors should send with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Journal of Physiology* but will be scrutinized by the Editors before being passed for publication in *International Abstracts*. It should contain a brief outline of the results and definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet, and should be headed as follows: Title of paper; name(s) of author(s); *J. Physiol., Lond.*, followed by space for year, volume and page reference; abbreviated postal address including country of origin. For example:

A quantitative description of membrane current and its application to conduction and excitation in nerve. A. L. Hodgkin and A. F. Huxley. *J. Physiol., Lond.*, 1952, **117**, 500–544 (Physiol. Lab., Univ. Camb., Cambridge, England).—Text of abstract follows straight on.

The short title *J. Physiol., Lond.*, is used in the abstract to avoid confusion with *J. Physiol., Paris*, but should not be employed in the reference lists of papers submitted to the Journal.

# THE BIOCHEMICAL JOURNAL

*Vol. 79, No. 3* **CONTENTS** *June 1961*

The biosynthesis of *N*-phosphorylcreatine: an investigation of the postulated alternative pathway. By J. F. Morrison and M. D. Doherty.

Tricarboxylic acid-cycle activity in *Streptomyces olivaceus*. By P. K. Maitra and S. C. Roy.

An amino acid sequence in the active centre of phosphoglucomutase. By C. Milstein and F. Sanger.

A native cobalamin–polypeptide complex from liver: amino acid composition and terminal amino acid analyses of the peptide part. By A. Hedbom.

The mechanism of the glyoxalase I reaction, and the effect of ophthalmic acid as an inhibitor. By E. E. Cliffe and S. G. Waley.

Studies in detoxication. 86. The metabolism of $^{14}$C-labelled ethylene glycol. By P. K. Gebbner, D. V. Parke and R. T. Williams.

Induction and repression of β-galactosidase in non-growing *Escherichia coli*. By J. Mandelstam.

Studies on the mode of action of excess of vitamin A. 1. Effect of excess of vitamin A on the metabolism and composition of embryonic chick-limb cartilage grown in organ culture. By J. T. Dingle, J. A. Lucy and H. B. Fell.

Studies on the mode of action of excess of vitamin A. 2. A possible role of intracellular proteases in the degradation of cartilage matrix. By J. A. Lucy, J. T. Dingle and H. B. Fell.

Studies on the mode of action of excess of vitamin A. 3. Release of a bound protease by the action of vitamin A. By J. T. Dingle.

A method for the chromatographic detection of penicillins and related compounds and of penicillinase. By P. H. A. Sneath and J. F. Collins.

Purification of latent phenolase from broad-bean (*Vicia faba*) leaves. By J. L. Bailey.

An enzyme from rat liver catalysing conjugations with glutathione. By J. Booth, E. Boyland and P. Sims.

The intrinsic viscosity of myosin and the interpretation of its hydrodynamic properties. By P. Johnson and A. J. Rowe.

Studies on the metabolism of the Chrysophyceae. Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. By A. Beattie, E. L. Hirst and E. Percival.

The effect of succinate and amytal on the reduction of acetoacetate in animal tissues. By H. A. Krebs, L. V. Eggleston and A. D'Alessandro.

Oligosaccharides in dextran-producing cultures of *Streptococcus bovis*. By E. J. Bourne, D. H. Hutson and H. Weigel.

The effect of hind-limb ischaemia on the products and intermediates of glycolysis in rat tissues. By C. J. Threlfall and H. B. Stoner.

The degradation of cotton cellulose by the extracellular cellulase of *Myrothecium verrucaria*. By K. Selby.

Folic acid and the synthesis of methionine by extracts of *Escherichia coli*. By K. M. Jones, J. R. Guest and D. D. Woods.

On the mechanism of activation of phosphoglucomutase by metal ions. By C. Milstein.

The mechanism of activation of phosphoglucomutase by chelating agents. By C. Milstein.

Inhibition of phosphoglucomutase by trace metals. By C. Milstein.

The effect of insulin on amino acid metabolism. By T. Hernandez and R. A. Coulson.

The effect of testicular hyaluronidase on the hyaluronic acid complexes of human synovial fluid. By D. Platt, W. Pigman and H. L. Holley.

The control of some oxidative pathways in guinea-pig mammary-gland mitochondria. By E. A. Jones and H. Gutfreund.

Biosynthesis of $C_6$ acids in *Pseudomonas fluorescens* KB1. By C. H. Wang and G. J. Ikeda.

The metabolism of naphthalene and 1-naphthol by houseflies and rats. By L. C. Terriere, R. B. Boose and W. T. Roubal.

Further purification and properties of the amine oxidase of pea seedlings. By P. J. G. Mann.

The liver-lipid constituents of male and female rats. 1. Effects of the fat-deficiency syndrome. By R. A. Morton and A. A. Horner.

The liver-lipid constituents of male and female rats. 2. Effects of the fat-deficiency syndrome, aggravated by dietary cholesterol. By A. A. Horner and R. A. Morton.

Ester-linked long-chain fatty acids of nervous tissue. By R. W. R. Baker.

The composition of collagen from subcellular fractions of guinea-pig granuloma tissue. By J. E. Eastoe.

*Appendix*—A semi-micro method for the determination of amino acids by ion-exchange chromatography. By J. E. Eastoe.

Some properties of coupled iron–caeruloplasmin oxidation systems. By G. Curzon.

Inactivation of arginine vasopressin by rat-kidney slices. By M. W. Smith and H. Sachs.

Acidic peptides of the lens. 7. The preparative enzymic synthesis of ophthalmic acid. By E. E. Cliffe and S. G. Waley.

*Proceedings of The Biochemical Society.*

*Subscription price per volume 80s. net. $13.50 in U.S.A.*

**CAMBRIDGE UNIVERSITY PRESS**

**BENTLEY HOUSE, 200 EUSTON ROAD, N.W.1**

## PHYSIOLOGY OF THE SALIVARY GLANDS

**A. S. V. Burgen,** M.D., M.R.C.P.
*Professor of Physiology, McGill University*

**N. G. Emmelin,** M.D.
*Professor of Physiology, University of Lund, Sweden*

No. 8 of the Monographs of the Physiological Society:

An account of the latest work on salivary gland function by two eminent scientists from Canada and Sweden. It includes chapters on Theories of Secretion; Metabolism; Physiological Role of Saliva; and Comparative Physiology of the Salivary Glands.

35*s.* net

**EDWARD ARNOLD (Publishers) LTD.**
**41 Maddox Street, London, W. 1**

*Particulars of the*

# *JOURNALS*

*Published by the Cambridge University Press, may be had from*

THE MANAGER
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD
LONDON, N.W. 1



## THE JOURNAL OF EXPERIMENTAL BIOLOGY

*Edited by* V. B. WIGGLESWORTH *and* J. A. RAMSAY

*Vol. 38, No. 2* CONTENTS *June 1961*



MOHRI, H. and HORIUCHI, K. Studies on the respiration of sea-urchin spermatozoa. III. Respiratory quotient.

DADD, R. H. Evidence for humoral regulation of digestive secretion in the beetle, *Tenebrio molitor*.

FISHER, R. C. A study in insect multiparasitism. I. Host selection and oviposition.

BEAMENT, J. W. L. The waterproofing mechanism of arthropods. II. The permeability of the cuticle of some aquatic insects.

RIEGEL, J. A. The influence of water-loading and low temperature on certain functional aspects of the crayfish antennal gland.

NIELSEN, BODIL. On the regulation of the respiration in reptiles. I. The effect of temperature and $CO_2$ on the respiration of lizards (*Lacerta*).

TREHERNE, J. E. Sodium and potassium fluxes in the abdominal nerve cord of the cockroach, *Periplaneta americana* L.

MACFADYEN, A. A new system for continuous respirometry of small air-breathing invertebrates under near-natural conditions.

ROBSON, ELAINE A. Some observations on the swimming behaviour of the anemone *Stomphia coccinea*.

OSBORNE, M. F. M. The hydrodynamical performance of migratory salmon.

RICE, A. L. The responses of certain mysids to changes in hydrostatic pressure.

ALEXANDER, R. MCN. The physical properties of the swimbladders of some South American Cypriniformes.

PARRY, GWYNETH. Osmotic and ionic changes in blood and muscle of migrating salmonids.

CRISP, D. J. Territorial behaviour in barnacle settlement.

LLOYD, R. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (*Salmo gairdnerii* Richardson).

ROWELL, C. H. FRASER. The structure and function of the prothoracic spine of the desert locust, *Schistocerca gregaria* Forskål.

WILSON, DONALD M. The central nervous control of flight in a locust.

RIEGEL, J. A. and LOCKWOOD, A. P. M. The role of the antennal gland in the osmotic and ionic regulation of *Carcinus maenas*.



*Subscription 84s. per volume, single parts 30s. net. Orders may be placed through your bookseller or direct with the*

***Cambridge University Press, Bentley House, 200 Euston Road, N.W.1***

# THE JOURNAL OF PHYSIOLOGY


The subscription price, for orders placed in advance of publication, for vols. 157, 158, 159 (1961) is 80*s.* per volume of 3 parts post free ($13.50 in the U.S.A.).

In normal circumstances, five volumes appear in each year.

Orders may be placed with your Bookseller, or sent direct to the

**Cambridge University Press,**
**Bentley House, 200 Euston Road, London, N.W. 1**

Also available from the **Cambridge University Press**

Back numbers from vol. 61 (1926) onwards.

Volumes 61–115, price £4. 10*s.* per volume.

Volumes 116–120, price £4 per volume.

Volumes 121–130, price £3. 15*s.* per volume.

Volumes 131–156, price £4. 10*s.* per volume.

Single issues and complete sets can be supplied.

Author and subject indexes to the Journal of Physiology, prepared by Dr Grace Eggleton.

Vols. 61–100. 30*s.* net.

Vols. 101–115. 30*s.* net.

Available from **Wm. Dawson & Sons, Ltd.**
**16 West Street, Farnham, Surrey, England**

Back numbers, vols. 1–25. Price £6 per volume.
Members' rates on application.

Ready shortly, vols. 20–60 bound, £8 per volume.

Some back issues and volumes are available from Volumes 1–60, particulars on request.

Author index of the Journal of Physiology, vols. 1–60. 25*s.* net.

Subject index of the Journal of Physiology, vols. 1–60, prepared by Dr J. G. Priestley. 15*s.* net.

History of the Physiological Society during its first fifty years 1876–1926. By Sir Edward Sharpey-Schafer, F.R.S. 15*s.* net.

*J. Physiol.* (1961), **157**, *pp.* 415–425
*With 2 text-figures*
*Printed in Great Britain*



# THE CHLORIDE CONTENT OF RAT AURICLE

By J. F. LAMB

*From the Department of Veterinary Physiology, University of Edinburgh*

(*Received* 28 *November* 1960)

In frog skeletal muscle (Adrian, 1960) and invertebrate muscle (Shaw, 1955), analysis has shown that the intracellular concentration of chloride is of the value expected if the ions were passively distributed around the resting membrane potential. This view was further strengthened by electrical experiments by Hodgkin & Horowicz (1959) and by Adrian (1960) on frog skeletal muscle, who showed that the membrane potential behaves in the way expected for passive distribution of chloride.

In cardiac muscle, on the other hand, there is less evidence on the chloride distribution. In the older analytical literature, e.g. Lohmann & Weicher (1934), Clark, Eggleton, Eggleton, Gaddie & Stewart (1938), it was shown that heart muscle contained more chloride than skeletal muscle, but this was difficult to interpret in the absence of simultaneous extracellular space measurements. More recent analyses by Robertson & Peyser (1956) and Barclay, Hamley & Houghton (1959), while showing a wide scatter of values, suggested that the intracellular levels of chloride were quite high. This was supported by the electrical experiments of Hutter & Noble (1959), which suggested that the chloride equilibrium potential was appreciably less than the maximum diastolic potential.

In the present experiments it will be shown that there is a considerable amount of chloride in heart cells, corresponding to an equilibrium potential of about 47 mV. A preliminary account of some of these experiments has already been given (Lamb, 1960*a*, *b*), but some of the conclusions reached in these papers were incorrect.

## METHODS

The animals used were 5-months-old Wistar Albino Glaxo rats, mostly female but with a few males. They were fed on ordinary diet and not starved before the experiments.

The general experimental procedure was to anaesthetize the animal with an intraperitoneal injection of urethane, remove the heart and then dissect free the left auricle in a paraffin-wax bath in Tyrode solution at 6° C. The auricle was then either suspended from a silver frame in Tyrode solution at 37° C in a test-tube (and hence left quiescent), or placed in an experimental bath in Tyrode solution at 35° C and driven at 1/sec, and action potentials recorded

with an intracellular electrode and conventional amplifying equipment. In a few experiments the auricles were snipped off the intact hearts and then analysed, with no contact with Tyrode solution.

*Solutions*

The NaCl Tyrode solution had the following composition (m-mole/l.): Na 149·3; K 2·7; Ca 1·8; Mg 1·05; Cl 145·4; $HCO_3$ 11·9; phosphate 0·4; glucose 5·56. The NaBr Tyrode solution was prepared by replacing the NaCl in this basic solution by an equivalent amount of NaBr. The choline chloride solution contained (m-mole/l.) choline 160, calcium 1·8, chloride 163·6, and atropine sulphate 1 mg/l. All solutions were equilibrated with 95 % $O_2$ and 5 % $CO_2$ during use.

*Chloride analysis*

The method used for the analysis was suggested by P. Croghan (see Ramsay, Brown & Croghan, 1955), and was an electrometric method. Initial experiments on the recovery of the chloride from the tissue showed that the greatest recovery was obtained when the hearts were soaked in 5 % nitric acid in the cold, and that dry ashing (no acid added) at temperatures of 300–500° C produced chloride losses of 20–50 %. Recovery experiments with NaCl only in a crucible showed no loss at 300° C.

After blotting the auricles, they were placed in crucibles 22 mm in diameter and height and then dried at 60° C for 0·5 hr. To each crucible was added 0·3 ml. of a 5 % solution of nitric acid, and the hearts were then allowed to stand overnight (18 hr) in the refrigerator. Next day the crucibles were removed and allowed to reach room temperature, and the chloride was then estimated directly in the crucible.

*Extracellular space measurement*

*Sucrose spaces.* An isotonic solution of sucrose (9·64 g/100 ml.) was prepared and kept in the refrigerator. Tyrode solution without the glucose was mixed in a proportion of 1 part of the sucrose solution to 9 parts of Tyrode, giving a final sucrose concentration of about 1 %.

Auricles were soaked in this solution in the usual way for 20–25 min (Johnson, 1957) and were then removed, blotted and placed in 5 ml. crucibles. They were dried at 60° C for 0·5 hr and weighed as usual. To each crucible was added 0·5 ml. of water, and the hearts were allowed to soak in this for 0·5 hr.

The sucrose content of the supernatant was then measured by the method of Heyrovsky (1956) after the auricle had been removed. (The method consists of adding b-indolyl acetic acid and concentrated HCl and then incubating the mixture in a water-bath at 35° C for 70 min. The resulting colour was read on a Hilger Spectrophotometer at 5300 Å.) It was found to be essential to run a heart blank with each batch of auricles, the necessary correction reducing the apparent sucrose content by about 15 %.

*Sodium wash-out technique*

The principle of the method (i.e. that at low temperatures it was possible to separate the efflux components due to intracellular sodium from those due to the extracellular sodium) was suggested by Dr A. M. Shanes and (separately) by Dr O. F. Hutter in conversation, and was adapted from a method used by Krnjević (1955) for the sciatic nerves of cats. In principle the hearts were washed with an isotonic solution of choline chloride at 5° C for varying periods, and then analysed for their sodium content.

*$^{82}$Br experiments*

$^{82}$Br was obtained from Harwell in the form of the ammonium salt. This was dissolved in distilled water and an equivalent quantity of NaOH was added. An appropriate amount of this solution was then placed in a test tube and dried, and 10 ml. of Tyrode solution was added. The exact technique of this preparation varied with the activity of the $^{82}$Br. When very active the Na$^{82}$Br was added to the normal Tyrode solution, thus increasing the sodium

and halogen content by about 1–2 %. When less active, an equivalent quantity of NaCl was omitted from the Tyrode diluting medium. The amount of Br introduced into the NaCl Tyrode solution in this way never exceeded 5 % of the total halogen. The final activity of this solution was about 20 $\mu$c/ml.

For the $^{82}$Br efflux experiments the auricles were soaked in this solution at 37° C for 1 hr, and then transferred through a series of test tubes containing 5 ml. of inactive Tyrode solution. The activity of the fluid in each test-tube, that of the soak-in fluid, and that remaining in the auricle was then measured with a well scintillation counter and scaler. All the efflux experiments were done at a temperature of 3–4° C (see Results section).

The influx experiments were done by soaking the auricles in the active solution, either at 37° C or at 4° C, for various times, and then following the efflux in the usual way, but for a shorter time.

Two experiments were done in which $^{82}$Br was injected into the intact rat, and the efflux from the auricle then followed in the usual way. For this purpose 1 mc of Na $^{82}$Br was injected into the peritoneal cavity of each rat (anaesthetized) and the animal left for 2 hr (no urine was passed during this time). The abdomen was then opened, a sample of blood was removed and spun down for the serum, and the auricle was then snipped off and the efflux started at 4° C in Tyrode solution. As the influx at 4° C (see Results) was negligible during the first minute, it seemed safe to assume that this procedure gave a measure of the $^{82}$Br introduced into the cells while the heart was in the animal and was not influenced by being immersed in Tyrode solution.

## RESULTS

*Extracellular space.* The value for this obtained by sucrose was $25{\cdot}1 \pm 0{\cdot}7$ (S.E. of mean) g/100 g wet weight (1135 g/kg dry weight) in ten auricles with a mean wet weight of $14{\cdot}2 \pm 0{\cdot}9$ mg and a water content of $77{\cdot}8 \pm 0{\cdot}4$ % (3540 g/kg dry weight). The value obtained by the sodium efflux method was 24·8 g/100 g wet weight, after the application of a correction for diffusion in the extracellular space (Dainty & Krnjević, 1955). These figures agree with that of 25·4 % found by Barclay *et al.* (1959) in perfused rat auricles; no other figures were found in the literature for rat auricle. It has been assumed that auricles with a water content of 78 % have an intracellular water content of 53 % (2410 g/kg dry weight) and an extracellular space of 25 %.

*Chloride analysis.* The chloride contents of groups of auricles under various conditions are shown in Table 1. Also shown is the mean value obtained for the serum chloride of four of the rats in group 1. This value for serum chloride is close to that of 110 m-mole/kg plasma found by Conway & Hingerty (1946). The main points demonstrated are as follows:

1. When the auricles are exposed to Tyrode's solution, there is an uptake of chloride of the order of 120 m-mole/kg dry weight, that is, about +70 % of the initial value.

2. This uptake occurs over the first 15 min of exposure in driven auricles but may take longer in quiescent auricles.

3. About 40 m-mole/kg dry weight of this chloride increase can be

accounted for in terms of the difference in the chloride content between plasma and Tyrode medium.

4. The rest of the increase, some 80 m-mole/kg dry weight, can only be accounted for by the increase in the total water content if this increase is all extracellular.

5. The calculated values of intracellular chloride from these results, assuming a constant intracellular water of 2410 g/kg dry weight, are 32 m-mole/kg fibre water in the living animals and 36 m-mole/kg fibre water in the soaked auricles (mean of 15 min and 1 hr).

TABLE 1. Table showing chloride content of auricles and plasma under various conditions. T represents normal Tyrode solution

| Treatment | No. of samples | Cl content (m-mole/kg dry weight) | S.E. of mean | Water content (g/kg dry weight) |
|---|---|---|---|---|
| No Tyrode | 7 | 176 | 5 | 3340 |
| 0·5 min T at 6° C | 4 | 225 | 4·4 | 3610 |
| 15 min T at 37° C (driven at 60/min) | 9 | 310 | 12·5 | 4030 |
| 120 min T at 37° C (quiescent) | 14 | 284 | 9·7 | 3680 |
| Plasma | 4 | 108 m-mole/kg plasma | 1 | Not measured |

Estimation of the intracellular chloride by this method is open to the criticism that the result is very dependent on the accuracy of the extracellular space measurement, and on whether the chloride and the sugar penetrate the same volume. It was, therefore, felt to be desirable to estimate the intracellular chloride in another way.

One suitable method is to analyse the wash-out curves of chloride from auricles, this giving a direct estimate of the amount of chloride in the intracellular compartment (see Shanes, 1957 for a general discussion of the technique as applied to nerve, and Dainty & Krnjević, 1955). Unfortunately chloride has no really convenient radioactive isotope, and use was therefore made of $^{82}Br$, which has a half-life of 36 hr. The justification for this was (1) that the total chloride, bromide and iodide contents of auricles under similar conditions are the same, (2) that the specific activity of four auricles in a NaCl Tyrode solution containing trace amounts of $^{82}Br$ was the same as the medium after soaking for 1 hr, and (3) wash-out curves of $^{82}Br$ into a NaBr Tyrode solution gave similar results to those into a NaCl Tyrode medium. Nevertheless, the use of $^{82}Br$ as a tracer for chloride can only give an indirect estimate of the intracellular chloride and therefore the term 'apparent chloride content' of the cells has been used.

*Tracer experiments.* In Fig. 1 is shown a typical wash-out curve for an auricle at 3° C plotted semi-logarithmically. It is seen that the initial fast

phase lasts some 10–20 min, and is then followed by a slower, nearly exponential portion. It may be assumed that the initial part of the curve represents bromide leaving the extracellular space, and that the slow part represents bromide leaving the cells (cf. Keynes, 1954; Shanes, 1957). If this is so, then the intracellular bromide content at the start of the wash-out can be estimated by extrapolation of the slow part of the curve to zero

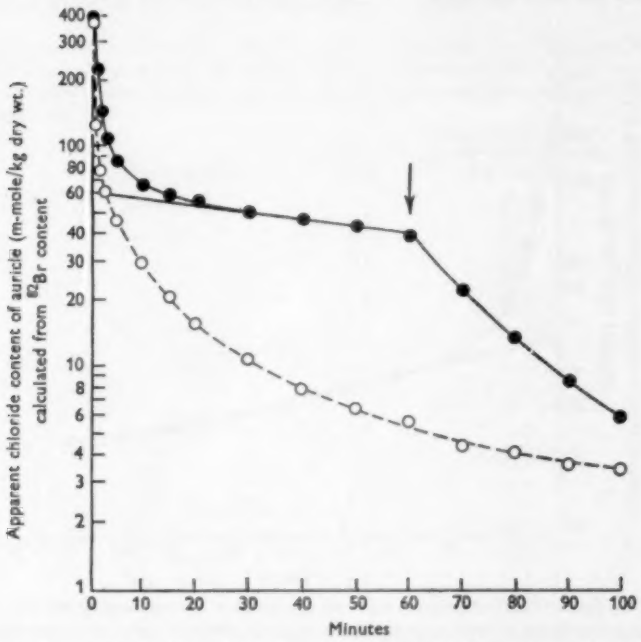



Fig. 1. Loss of labelled bromide from rat auricles at 3° C (upper curve) and 37° C (lower curve) after soaking in NaCl Tyrode solution containing trace amounts of $^{82}$Br at 37° C for 1 hr. At the arrow, temperature was increased from 3 to 37° C in the upper curve. Slow part of the upper curve fitted with a straight line by eye. Apparent chloride content of the tissues calculated from the specific activity of the medium, assuming that bromide and chloride are distributed in the same way. Error of counts ($\pm 2 \times$ S.E.) was less than the diameter of the symbol used for each point. Semi-log. scale.

time (Fig. 1). This can only be a rough estimate, as it has been shown by Dainty & Krnjević (1955) that the value has to be corrected for slow diffusion in the extracellular space. The amount of correction required depends essentially on the rate constants of the slow and fast components of the efflux curve. Unfortunately in these tissues the fast component obtained by subtracting the linear extrapolation from the original record

has a complicated form (see Keynes, 1954) and it is therefore not possible to apply the correction for diffusion in the extracellular space in any rigorous fashion; but if it is assumed that the initial half of this fast process is a fair approximation to the diffusion process then the correction required is only a few per cent. No correction has been applied to the linear extrapolations obtained in this paper, so that probably all the values obtained are a few per cent too high.

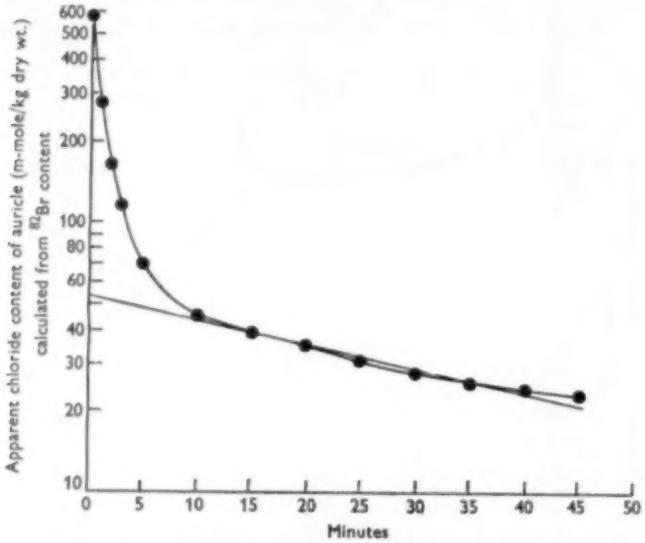



Fig. 2. Loss of labelled bromide from rat auricle at 3° C after loading the rat with radioactive bromide 2 hr before death. Straight line fitted to slow part by regression equation. Apparent chloride content of the auricle calculated from the specific activity of the plasma, assuming that bromide and chloride are distributed in the same way. Errors as in Fig. 1. Semi-log. scale.

With wash-out curves obtained at 37° C there were no separable components in eight out of ten experiments. In two cases, however, an efflux curve was obtained with a fast and slow component as at 3° C. In one of these experiments the efflux curve was repeated, with a similar result. As far as was known these auricles were treated in the same way as the others, and no reason can be given for this difference. Consequently, all the effluxes were measured at 3–4° C, where curves of the type shown in Fig. 1 were obtained consistently.

In Fig. 2 is shown one of the efflux curves from the auricles loaded with $^{82}$Br while still in the rat. From these and other similar curves, assuming

the distribution of Br and Cl to be identical, the amount of chloride in the cells of intact (beating) and soaked (quiescent) auricles has been calculated. The values obtained are shown in Table 2.

One experiment was done in which the left and the right auricles were left connected during the soak-in period, so that the left auricle was being driven at about 200/min. This auricle (Table 2) also had the same order of apparent chloride content as the others.

TABLE 2. Apparent intracellular chloride contents calculated from $^{82}$Br experiments. All wash-outs carried out at 3° C. All uptakes at 37° C except those indicated. All auricles were quiescent except those indicated. In lower part of table each auricle used twice. T represents Tyrode solution

| $^{82}$Br treatment | Dry weight (mg) | Apparent intracellular chloride (m-mole/kg dry weight) |
|---|---|---|
| 1 hr NaCl T | 3·1 | 64·6 |
| | 3·0 | 53·6 |
| | 3·5 | 67·9 |
| | 3·4 | 42·6 |
| | 2·4 | 59·5 |
| | 2·4 | 62·5 |
| | 2·7 | 48·7 |
| 1 hr NaCl T (beating at 200/min) | 1·6 | 67·7 |
| 1 hr NaBr T | 4·0 | 56·5 |
| | 4·5 | 71·0 |
| | Mean | 59·5 ± 2·8 (S.E.) |
| 1 min NaCl T | 2·8 | 43·7 |
| 5 min | — | 42·4 |
| 3 min | 2·4 | 70·8 |
| 8 min | — | 59·3 |
| 1 min NaBr T* | 2·2 | † |
| 6 min* | — | 19·8 |
| 3 min* | 3·0 | 5·0 |
| 10½ min* | — | 12·2 |
| 2 hr *in vivo* | 2·5 | 41·0 |
| | 1·9 | 53·0 |

* 4° C. † Not detectable.

*Uptake experiments.* So far it has been assumed that the cells came into equilibrium with the $^{82}$Br in the Tyrode in the hour's soak-in time. In Table 2 (lower part) are shown the apparent intracellular chloride values obtained after uptakes at 37 and 4° C for the times shown. It can be seen that after 1 min at 37° C the value reached is already within the range found after 1 hr uptake, so that one hour's soaking should be adequate for full equilibrium to be reached. Also, when checked in four experiments, the specific activity of the tissues equalled that of the soak-in fluid after one hour's soaking.

*Effect of temperature on fluxes.* Figure 1 shows the typical effect of temperature on the slow part of the efflux. Table 2 (lower part) also shows the effect of a temperature of 4° C on the uptake of $^{82}$Br by the cells. After 1 min no intracellular component could be detected and the greatest

value attained during the first 10 min of uptake at 4° C was less than half of the value expected at 37° C.

*Intracellular chloride level.* The mean apparent $[Cl]_i$ value in auricles soaked in Tyrode solution for 1 hr, taking the intracellular water content as 2410 g/kg dry weight, is, from Table 2, $25 \pm 1$ m-mole/kg fibre water. That for the two auricles removed from the rat after loading *in vivo* is 19·5 m-mole/kg fibre water, calculated for the same intracellular space as the soaked auricles. Clearly, then, the intracellular chloride values determined in this way are similar whether the auricles are soaked in Tyrode solution or not.

TABLE 3. Distribution of chloride (m-mole/kg dry wt.) in freshly dissected and soaked auricles. The measured extracellular space of soaked auricles is 1135 g/kg dry weight

| | Total Cl | Apparent intracellular chloride | Calculated extracellular chloride | Extracellular water required to hold this chloride |
|---|---|---|---|---|
| Freshly dissected auricles | 176 | 47 | 129 | 1195 (at 108 mM) |
| Tyrode-soaked auricles | 284 | 60 | 224 | 1540 (at 145 mM) |

For soaked and non-soaked auricles both the apparent intracellular chloride and the total chloride contents are known, so that the chloride in the extracellular space can be obtained from the difference, and hence the space required to hold it. These calculations are shown in Table 3. The observed extracellular space in soaked auricles is 1135 g/kg dry weight, which is some 35 % lower than that required to hold all the chloride. As the extracellular space measured by two different methods, and that found in the literature, all agree to within 2 %, it seems unlikely that an error of 30 % could be present.

## DISCUSSION

*Chloride uptake.* The results presented here show that auricles exposed to Tyrode solution take up about 70 % more chloride than was originally present in them. This uptake takes place in the first 15 min in auricles driven at 1/sec, but may take longer in quiescent auricles. In the present experiments the auricles also took up water on soaking in Tyrode solution, and as the extracellular space was only measured in the soaked preparation it is not known which compartment this water entered. If it is assumed that all the water increase was extracellular (as suggested by the results of Hercus, McDowall & Mendel, 1955) then nearly all the extra chloride can be accounted for in the extracellular space. On the other hand, if some or nearly all the extra water entered the cells, then this extra chloride cannot be accounted for in this simple way. Evidence in favour of this latter view is shown in Table 3, in which the calculated space required to hold the extracellular chloride in freshly dissected auricles is within 5 % of

that estimated in the soaked auricles, whereas the calculated extracellular space for soaked auricles is some 35% higher than that observed under the same conditions. If these figures are correct they suggest that chloride in soaked auricles becomes distributed in a larger extracellular space than is available to the sucrose and sodium.

In frog sartorius Eggleton, Eggleton & Hamilton (1937) showed that the chloride 'space' increased from about 14% in the body to 30% in soaking in Ringer's solution or on perfusing the limb with Ringer's solution. The size of the chloride space in the living frog agrees well with the usually accepted value for the extracellular space of soaked sartorii (Boyle & Conway, 1941; Desmedt, 1953), so that the increase of the total chloride is unlikely to be due to a large extracellular space. Recently Adrian (1960) has reported low figures for intracellular chloride in soaked sartorii (measured by analysis of $^{36}Cl$ efflux curves), thus making it unlikely that the extra chloride enters the cells. It seems likely, then, that in skeletal as in heart muscle there is an uptake of chloride on exposure to a salt solution and that this uptake cannot be accounted for in simple terms.

*Intracellular chloride concentration.* Because of this uncertainty about the distribution of the extra chloride, it is probable that values of intracellular chloride obtained by calculation from the total chloride and the extracellular space in heart and skeletal muscle are unreliable. Thus the figures given by Lamb (1960*a*) for the effect of DNP on the chloride content of rat auricle do not now provide any evidence for the active transport of chloride.

With the assumption that bromide and chloride are distributed in a similar way in heart, the apparent intracellular chloride contents can be used to calculate the equilibrium potential for chloride. In intact auricles in the rat the intracellular chloride is 19·5 m-mole/kg fibre water and the plasma chloride 108 m-mole/l., giving an equilibrium potential for chloride of 46 mV. The corresponding figures for soaked auricles are 25 m-mole/kg fibre water and 145 m-mole/l. Tyrode solution, with an equilibrium potential of 47 mV. These experiments probably over-estimate the intracellular chloride, and if for example the true values are 10% lower, then these equilibrium potentials would be about 3 mV greater.

In the living animal the maximum diastolic potential is likely to be about 80 mV ($80 \pm 0{\cdot}9$ mV in Tyrode solution, Lamb 1960*b*), with action potentials superimposed on it at a rate of 300/min, each lasting about 50 msec. This means that the membrane potential will be less than the maximum diastolic potential for about a quarter of the total time, and therefore it is possible (depending on the permeability to chloride during the action potential) that chloride is passively distributed around a potential less than the maximum diastolic one, in the living animal.

It is not known with certainty what the resting potential in the soaked quiescent preparation was, there being no direct evidence on this point in these experiments. If heart is like skeletal muscle, then lack of activity will not prevent the resting potential from being close to 80 mV. If this is so, then the expected $[Cl]_i$ will be 7 m-mole/kg fibre water, a value about a third of that observed.

In view of these considerations it is not possible, on the present evidence, to say whether or not the apparently high value of $[Cl]_i$ in rat auricle (and presumably other heart tissues) is consistent with passive distribution across the membrane or due to active inward transport of chloride. It seems reasonably clear, however, that the chloride equilibrium potential is substantially less than the maximum diastolic potential and, as this is maintained during the hour's quiescence despite a high rate of exchange across the membrane, it suggests that the normal repetitive activity of the heart has little influence on the distribution of chloride.

## SUMMARY

1. Experiments have been done with rat auricles, removed direct from the animals and also soaked in Tyrode solution. Measurements of extracellular space, total chloride and apparent intracellular chloride (by $^{82}Br$ efflux) have been carried out.

2. Rat auricles take up some 70% more chloride on exposure to Tyrode solution than they contain in the intact animal.

3. This extra chloride cannot easily be accounted for on the basis of an increased extracellular space, nor by an increase of the intracellular chloride content.

4. In the intact animal the plasma chloride is 108 m-mole/kg and the apparent intracellular chloride 19·5 m-mole/kg fibre water. In the Tyrode-soaked auricles the intracellular chloride rises to 25 m-mole/kg fibre water with an extracellular chloride of 145 m-mole/kg. The equilibrium potential for chloride in these two states is therefore about 47 mV.

I am very indebted to Dr J. R. Greening and the staff of the Medical Physics Unit, 12 George Square, Edinburgh, for help and facilities given during these experiments; and to Dr P. C. Croghan for instruction in the method of chloride analysis.

## REFERENCES

Adrian, R. H. (1960). Potassium chloride movement and the membrane potential of frog muscle. *J. Physiol.* **151**, 154–185.

Barclay, J. A., Hamley, E. J. & Houghton, H. (1959). The inulin, sucrose, chloride and sodium space of the isolated perfused rat heart. *J. Physiol.* **149**, 15–19*P*.

Boyle, P. J. & Conway, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1–63.

Clark, A. J., Eggleton, M. G., Eggleton, P., Gaddie, R. & Stewart, C. P. (1938). *The Metabolism of the Frog's Heart.* Edinburgh: Oliver and Boyd.

CONWAY, E. J. & HINGERTY, D. (1946). The influence of adrenalectomy on muscle constituents. *Biochem. J.* **40**, 561–568.

DAINTY, J. & KRNJEVIĆ, K. (1955). The distribution of Na and K in cat nerves. *J. Physiol.* **128**, 473–488.

DESMEDT, J. E. (1953). Electrical activity and intracellular sodium concentration in frog muscle. *J. Physiol.* **121**, 191–205.

EGGLETON, M. G., EGGLETON, P. & HAMILTON, A. M. (1937). Distribution of chloride in frog's skeletal muscle immersed in saline solution. *J. Physiol.* **90**, 167–182.

HERCUS, V. M., MCDOWALL, R. J. S. & MENDEL, D. (1955). Sodium exchanges in cardiac muscle. *J. Physiol.* **129**, 177–183.

HEYROVSKY, A. (1956). A new method for the determination of inulin in plasma and urine. *Clin. Chemica Acta*, **1**, 470–474.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HUTTER, O. F. & NOBLE, D. (1959). The influence of anions on impulse generation and membrane conductance in Purkinje and myocardial fibres. *J. Physiol.* **147**, 16–17 *P*.

JOHNSON, J. A. (1957). Sodium exchange in the frog heart ventricle. *Amer. J. Physiol.* **191**, 487–492.

KEYNES, R. D. (1954). The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B, **142**, 359–382.

KRNJEVIĆ, K. (1955). The distribution of Na and K in cat nerves. *J. Physiol.* **128**, 473–488.

LAMB, J. F. (1960*a*). 2, 4-Dinitrophenol and the chloride content of rat auricle. *J. Physiol.* **152**, 31–32 *P*.

LAMB, J. F. (1960*b*). *Electrical activity and ionic gradients in cardiac muscle, with special reference to the effects of* 2·4-*dinitrophenol.* Ph.D. Thesis, University of Edinburgh.

LOHMANN, K. & WEICHER, B. (1934). *Oppenheimers Handbuch der Biochemie des Menschen und der Tiere.* 2nd ed. Ergänz. Vol. II (Suppl. Vol. IV–VI) pp. 245–248. Jena: Gustav Fischer.

RAMSAY, J. A., BROWN, R. H. J. & CROGHAN, P. C. (1955). Electrometric titration of chloride in small volumes. *J. exp. Biol.* **32**, 822–829.

ROBERTSON, W. V. B. & PEYSER, P. (1956). Estimates of extracellular fluid volume of myocardium. *Amer. J. Physiol.* **184**, 171–174.

SHANES, A. M. (1957). Ionic transfer in a vertebrate nerve. In MURPHY, Q.R. *Metabolic Aspects of Transport Across Cell Membranes.* Madison: University of Wisconsin Press.

SHAW, J. (1955). Ionic regulation in the muscle fibres of Carcinus maenus. *J. exp. Biol.* **32**, 383–396.

*J. Physiol.* (1961), **157**, *pp.* 426–453
*With 9 text-figures*
*Printed in Great Britain*

# THE RELATION OF PULSATILE PRESSURE AND FLOW IN THE PULMONARY VASCULAR BED

By C. G. CARO* and D. A. McDONALD
*From the Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

(*Received* 16 *January* 1961)

The flow of blood in the pulmonary artery is pulsatile but it can be analysed, as can systemic arterial flow (McDonald, 1960), by resolving it into two components. These are a steady, or mean, forward flow throughout the cardiac cycle and an oscillatory flow which fluctuates about that mean. The ratio of the mean pressure drop between the pulmonary artery and the left atrium to the mean flow has been extensively studied and is defined as the vascular resistance. By a similar analogy with the electrical terms for alternating current, the relationship between the oscillatory pressure and the oscillatory flow at the origin of the pulmonary trunk may be described in terms of the fluid input impedance.

This latter concept has been developed at length in previous reviews (McDonald & Taylor, 1959; McDonald, 1960). The term input impedance can only be precisely applied to a simple harmonic oscillation at a given frequency. Thus, if the pressure and flow created by the heart are measured, a Fourier, or harmonic, analysis of both curves is necessary. In the series of experiments reported here we have imposed sinusoidal oscillations, generated by a pump, on to a steady flow in perfused rabbit lungs. While more artificial than the study of flow from the heart, the technique has enabled us to make measurements with greater ease and precision over a more varied range of frequencies.

Preliminary communications on these topics have been presented to the Physiological Society; that is, on wave velocity (Caro & McDonald, 1960) and on the input impedance (Bergel, Caro & McDonald, 1960).

## METHODS

A series of twenty-four rabbits (2·3–4·6 kg) were anaesthetized with I.V. pentobarbitone (Nembutal, Abbott Laboratories; 30 mg/kg body wt.) and heparinized (1 ml. Roche Liquemin containing 5000 i.u.). A rib was resected and the thorax widely excised. The animal was bled to death. For perfusion of the pulmonary bed a somewhat angled glass

* Present address: Department of Medicine, St Thomas's Hospital Medical School, S.E. 1.

cannula was introduced into the pulmonary artery through an incision in the right ventricle; it was held by a ligature just distal to the pulmonary valves. The cannula had a side arm (bore *ca.* 3 mm) which was connected to a manometer. A second cannula was inserted into the left atrium through an incision in the left ventricle and connected to a constant-level reservoir. Normally this was held so that the atrium was at atmospheric pressure. A diagram of the apparatus is shown in Fig. 1. Perfusion was carried out with isotonic saline solution in nearly all experiments. In some, Dextran (Dextraven, Bengers Ltd.) was substituted in an effort to retard the onset of pulmonary oedema, but was abandoned because it was without effect.

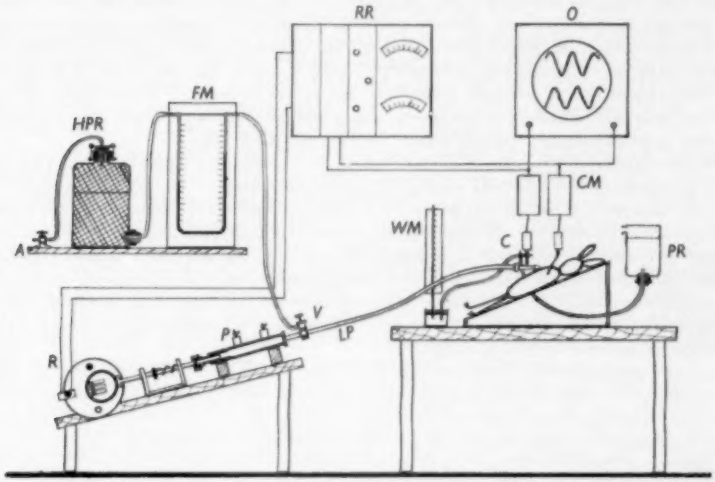



Fig. 1. A diagram of the apparatus used for measuring the input impedance and the wave-velocity in the pulmonary arterial bed of a rabbit. *A* compressed air; *C* cannula; *CM* capacitance manometers; *FM* flowmeter; *HPR* high-pressure perfusion reservoir; *LP* lead pipe; *O* oscilloscope; *P* pump; *PR* perfusion reservoir; *R* reservoir; *RR* resolver relays, controls and meters; *RV* reducing valve; *WM* water manometer.

*Oscillatory flow.* This was generated by a cam-driven sinusoidal pump designed by Taylor (1957*b*, 1959), using the barrel of a 5 ml. all-glass syringe. The output is known to contain not more than 2–3 % of second harmonic. Pump frequencies were held steady by the use of a velodyne motor. With 4:1 reduction gearing the frequency range studied was from 3 to 20 c/s, and a later change enabled us to study the range 1–13 c/s; at frequencies below 3 c/s a flywheel on the motor shaft improved the evenness of running. The stroke volume was varied between 0·08 and 0·72 ml. in various experiments. The linear displacement of the piston was measured by means of a vernier caliper; a calibration curve relating this to the volume displacement was made with a micro-burette, and shown to be a straight line.

In order to ensure that the oscillatory flow created by the pump was identical to that at the pulmonary cannula the two were connected by a 1 m length of lead piping (ext. diam. 12·5 mm; wall thickness 2 mm) with brass adaptors at either end. To reduce the likelihood of air bubbles becoming trapped the apparatus was cleaned with Teepol each day and the animal was arranged on a stand so that the pipe ran up at an angle of about 25° throughout. Any bubbles moved by flushing therefore became visible in the glass pulmonary cannula.

The pump assembly was made of Perspex and contained taps where bubbles could be removed. These precautions are important, for an air bubble in the lead pipe can markedly alter the amplitude and phase of the flow at the cannula relative to that created by the pump. Bubbles close to the manometer will, of course, introduce marked distortion in the pressure recording. At the higher frequencies pressures which are subatmospheric may easily be created at the pump and air sucked in—even when the joints appear to be tight.

*Steady flow.* Steady perfusion was created from a 10 l. glass reservoir enclosed in expanded metal and kept at an absolute pressure of 2 atm from a compressed-air source. This was linked to the lungs through a screw-reducing valve (Bassett Lowke) at a proximal end of the lead piping. The large pressure drop across this valve was designed to ensure that no oscillatory flow passed into the reservoir. Steady flow was measured by recording the pressure drop across a capillary tube (length 22 cm; bore 2 mm) inserted in the high-pressure line. This pressure drop was measured by a mercury U-tube sealed to the two ends. A calibration curve was made of the pressure difference against known volume flows—owing to inlet length effects this relation was not linear. In nearly all experiments flow was adjusted so that the mean pressure in the pulmonary artery was 10 mm Hg.

The fact that no oscillations were observed on the steady-flow meter indicates that none of the pump-generated flow was leaking back into the reservoir. An additional check was made by measuring the input impedance of a length of rubber tube clamped at the far end. This was done first with the valve completely closed and then with the valve open the usual amount but with the reservoir at atmospheric pressure. No difference could be detected under these unfavourable conditions (for the impedance of the tube was higher than that of the pulmonary bed and the impedance of the valve would obviously be raised by subjecting the far side to an extra pressure of 1 atmosphere); hence it was assumed that no appreciable distortion of recorded values occurred.

A second small perfusion reservoir, whose height could be varied, was used for maintaining perfusion of the lungs during setting up and while refilling the high-pressure reservoir. This was connected to the side arm of the perfusion cannula. It was also used for manometer calibration in conjunction with a water manometer which could be switched into the circuit.

*Inflation of the lungs.* In the earlier experiments the lungs were inflated to a measured pressure and this was held constant throughout a run over the whole frequency range of the pump. When it was found that the impedance varied very little with the degree of inflation of the lungs, cyclical positive pressure inflation from a respiratory pump (Palmers Ltd) was substituted for static inflation. The impression was gained that the onset of oedema occurred later when cyclical inflation was used. Lung pressure was measured throughout with a water manometer.

### *Measurement of pressure*

As the rate of the oscillatory flow was determined by the stroke and frequency of the pump, the only variable that needed to be recorded, in order to measure the input impedance, was the pressure at the input into the pulmonary vascular bed. This was measured with a capacitance manometer (Southern Instruments Ltd) attached directly through a three-way metal tap (connecting with the subsidiary perfusion reservoir) to the wide glass side arm of the pulmonary cannula. As the 400 mm Hg head was normally used the resonant frequency of the manometer and connexions was about 400 c/s and the damping was about 0·02. The maximum frequency imposed was 20 c/s, therefore there was no appreciable distortion of either amplitude or phase. The sensitivity of the manometer was increased in all experiments with small stroke volumes by placing an additional amplifier between the manometer and resolver.

The output of the manometer was displayed on a Cossor 1049 two-beam oscilloscope but this was principally used as a monitor to ensure that there was no deviation from a sinusoidal wave form. Measurements of pressure were made through an harmonic resolver (Taylor,

1957*b*, 1959). In principle this depends on two half-wave rectifications of the wave by two two-pole chopper relays activated by two sets of contacts 180° apart on a wheel driven off the cam-shaft of the pump. The two sets of contacts were 90° out of phase with each other and two damped centre-reading meters then read the integral value of the cosine and sine Fourier components of the pressure wave. The method of calculating the modulus and phase of the impedance from these readings is discussed below. The resolver was arranged so that it could be switched to the input of a second manometer (when wave velocity was being measured); other contacts were fixed on the resolver wheel so that any second and third harmonics of the pump frequency could be detected and measured.

*Measurement of wave velocity*

The apparent phase velocity of a pressure wave is defined by the phase shift of a single harmonic component over a known distance. Pressure was measured, as described above, at the origin of the pulmonary artery and simultaneously with an additional capacitance manometer from a site in a peripheral pulmonary artery. By the use of the resolver the phase shift of the wave between these points could be determined.

The method of cannulating a peripheral pulmonary artery was as follows: A measured length of 1·0 or 1·5 mm bore stiff nylon tubing, threaded over a somewhat longer piece of catgut, was passed along the arterial bed until it wedged in a peripheral artery. The tubing was then forced through the lung surface and drawn out to the exterior until its proximal end lay at the point where it had wedged, when it was secured by an exterior ligature. The catgut was then seized at the pulmonary valves and pulled out of the arterial tree; measurement of its length minus the length of the nylon tube then gave the distance between the origin of the pulmonary artery and the proximal tip of the peripheral catheter. In the first two experiments on the wave velocity the distance was measured externally, but these values are not regarded as being so accurate as those measured with the catgut.

A second capacitance manometer was attached to the nylon tubing which was cut off at as short a distance as was feasible. Nevertheless the resonant frequency was less than that of the proximal manometer. With the stiffest membrane (400 mm Hg head) it was *ca.* 300 c/s or higher; with a less stiff membrane (200 mm Hg head) it was 150–180 c/s with a damping *ca.* 0·1 or smaller. This is still over ten times the highest frequency used to measure wave velocity, and so is regarded as tolerable (McDonald, 1960, Ch. 11). The expected phase error at 15 c/s might, however, be as much as 0·1 radians. As the measured phase shift at this frequency was often well over $2\pi$ radians no attempt was made to correct for phase error in the distal manometer.

*Pressure measurement in pulmonary veins*

A length of nylon tube, similar to that used in the arterial measurements, was passed through a small incision in the left atrial appendage into a pulmonary vein and advanced until its tip was seen to lie in the region of the confluence of the lobar veins. Pressure recording was the same as for recording in the peripheral arteries. The phase shift between the origin and the veins was recorded but phase velocities could not be determined, as the length of the path through the capillary bed could not be measured.

*Sources of error*

The most potent source of error in impedance measurements is the presence of air bubbles. The precautions taken to eliminate these have been described. Nevertheless, bubbles did occasionally lodge in the lead pipe. If this occurred during a run there was usually an unexpected change in the phase of the pressure readings, or possibly a drop in the amplitude of the pressure oscillation. Whenever bubbles were discovered the previous readings of the run were discarded.

A second source of error is introduced by the fact that the vascular bed is a non-linear system (Womersley, 1957). This is shown by the fact that a virtually pure sinusoidal input

gives rise to an appreciable amount of second and even third harmonic. This problem has only been investigated very superficially in the present work. The main reason for this was the great complication it would have added to the work. In addition, faults developed in the contacts for higher harmonics on the resolver. In practice we accepted any results in which the output appeared sinusoidal on the monitor CRO. This would tolerate up to 10% of second harmonic.

The development of higher harmonics is due in large part to the oscillatory dilatation of the vessels and hence to the amplitude of the oscillatory pressure developed. As the oscillatory pressure increases with frequency, with a constant stroke on the pump, these errors are much greater at the upper end of the range. In practice we found very little distortion provided that the amplitude of the oscillatory pressure did not exceed the mean pressure. In early experiments with relatively large stroke volumes the pressure swings reached over 150% of the mean pressure and the wave form became grossly distorted. The inclusion of these results appears to account for the larger scatter in the input impedance at high frequencies.

The way to obviate this was to use a much smaller stroke volume. This raises the converse difficulty that the pressure oscillations were very small at low frequencies, e.g. if they are $\pm 10$ cm $H_2O$ at 16 c/s then they may only be $\pm 0{\cdot}5$ cm $H_2O$ at 1 c/s. Such pressure oscillations were in fact measured with the aid of the additional amplifier in the manometer output. When measured with a manometer designed to work over the range 0–400 mm Hg this naturally raises a question of accuracy. In fact, both calibration studies and the fact that the reproducibility of results were best where the pressure readings were lowest (usually around 3–4 c/s) suggest that this manometer was recording these small oscillations accurately.

The calibration of the manometers has been discussed above. Taylor (1959) has also noted that the response time of the relays in the resolver, although only 1·9 msec, introduces an appreciable phase error—*ca.* 0·25 radians at 20 c/s. As the absolute phase was not considered in any detail this correction has not been made. In measuring wave velocity these phase errors will be the same for each recording point and hence not appear in the phase difference. The principal source of error here is in measuring the distance between the recording points. The methods used for doing this have been detailed above. Estimates made by the catgut method and external measurement might differ by as much as 1 cm and thus in a 4 cm pathway might cause a 25% error. In practice the value obtained by the catgut method was accepted. In the two experiments where external measurements only were used the results may well be 15–20% too high (they were the two highest velocities recorded).

The measured wave velocity will be the sum of the true wave velocity and the mean flow velocity (McDonald & Taylor, 1959). Over the main part of the arterial bed the mean velocity of flow is probably not more than 3–5% of the wave velocity; in the pulmonary trunk, however, flow velocity may be as much as 30% of the measured wave velocity and allowance for this would be needed.

The transmission of vibrations from the pump to the proximal manometer was very difficult to eliminate because of the rigid connexions. It was minimized by constructing all supports on the operating table of tubular scaffolding. The vibrations in the input pressure record were of a very much higher frequency than the pump oscillation and so were not recorded by the resolver. The manometer used to measure the peripheral arterial or venous pressure was suspended independently and as it was attached to a flexible catheter was not subject to vibration. Considerable care was taken to see that none of the small venous pressure oscillations were due to this cause by showing that not only were they not in phase with the input but that the phase lag increased progressively with frequency. Hence it was due to a transmission time.

*Calculation of results*

The units for impedance in this study have been dyn.sec/cm$^5$. That is the pressure (dyn/cm$^2$) divided by the volume flow (cm$^3$/sec). This unit has been adopted in spite of the reasons advanced by Taylor (1959) and McDonald (1960) for using the ratio of pressure to average linear velocity of flow—making the unit dyn.sec/cm$^3$. This has been done because of the difficulty of measuring the true diameter of the pulmonary trunk with a glass cannula in it, in order to derive the linear velocity from the measured volume flux. Cotton (1960) has also argued that the volume flux is a better analogue of electrical current than is velocity. Either system is internally consistent.

The rate of volume flow ($Q$) is determined by the amplitude of the pump oscillation ($V$) and the frequency ($f$ c/s). The amplitude means the maximum deviation from the mean position, i.e. the pump puts out $+V$ ml. on forward stroke and withdraws to $-V$ ml. Under these circumstances

$$|Q| = 2\pi f \times V, \tag{1}$$

where $|Q|$ is the amplitude or modulus of the volume flux. As the measured stroke volume ($V_s$) is from maximum forward to maximum back, i.e. $2V$, for calculation

$$|Q| = \pi . f . V_s. \tag{2}$$

The pressure records made on the resolver are the integrated values of the half wave-rectified cosine and sine components. The values actually recorded on meters, $A'$ and $B'$, are related to the true amplitudes by the following equations (Taylor, 1957*b*):

$$A' = 2/\pi(A_1 + \tfrac{1}{3}A_3 + \tfrac{1}{5}A_5 \ldots), \tag{3}$$

$$B' = 2/\pi(B_1 + \tfrac{1}{3}B_3 + \tfrac{1}{5}B_5 \ldots), \tag{4}$$

where $A_1$, $A_3$ and $A_5$ are the Fourier cosine components of the first, third and fifth harmonics and $B_1$, etc., the corresponding sine terms. As we are dealing with a single harmonic oscillation the terms for the third and higher odd-numbered harmonics are negligible. Therefore to get the correct value of the Fourier terms $A_1$ and $B_1$ the meter readings $A'$ and $B'$ have to be multiplied by $\frac{1}{2}\pi$. They are also, of course, multiplied by a manometer calibration term to convert to actual pressure units.

Given the cosine and sine terms of the pressure this may be handled as a complex number but it is easier to convert into modulus and phase form (McDonald, 1960, App. 1). The amplitude, or modulus $M$ is given by Pythagoras's theorem

$$M = (A^2 + B^2)^{\frac{1}{2}}, \tag{5}$$

and the phase $\phi$ by

$$\phi = \tan^{-1} B/A. \tag{6}$$

The pressure, $P$, then is $M\cos(\omega t - \phi)$. It is necessary to determine the correct quadrant for $\phi$ by the signs of the cosine and sine terms, e.g. if both $A$ and $B$ are positive $\phi$ lies between 0° and 90° (0 and $\frac{1}{2}\pi$ radians); if $A$ is negative and $B$ positive it lies between 90° and 180° and the angle is $180° - \phi$. As the phase of the flow is fixed at 0° by the resolver setting, the phase of the pressure is also the phase of the input impedance.

As the modulus of the flow is simply determined by eqn. 2 the modulus of the impedance $|Z|$ is $|P|/|Q|$, or from eqns. 5 and 2

$$|Z| = M/(\pi . f . V_s). \tag{7}$$

To measure the wave velocity we have a known distance between measuring points, $\Delta x$. The modulus and phase are recorded at each point as described above. Then the difference in phase between the two points, $\Delta\phi$, represents the time (expressed as a fraction of a cycle) for the wave to travel between the two points. The apparent phase velocity $c'$ averaged over this interval then will be

$$c' = \frac{2\pi . f . \Delta x}{\Delta\phi} \tag{8}$$

when $\Delta\phi$ is measured in radians. If it is measured in degrees then 360 should be substituted for $2\pi$.

*The interpretation of these results* is made on precisely the same basis as that of oscillatory flow in the systemic arterial system (McDonald, 1960, Ch. 10). In a system of branching tubes the occurrence of wave reflexion is inevitable. In such a situation the input impedance will not only be determined by the calibre of the vessels and their elastic properties but also on the length of the system from the origin to the main reflecting sites. The unit of length that we are concerned with is the wave-length, i.e. the wave velocity divided by the frequency in c/s. The type of reflexion that we meet in a system subdividing into smaller branches is that known as 'closed-end', or 'in-phase'. This is the type of reflexion that occurs when the impedance increases across a junction (when completely closed, of course, the terminal impedance becomes infinite). As it is known that fluid resistance increases with subdivision of the vessels, so also does fluid impedance increase, although this effect is small in the large vessels and only becomes marked in small arteries and arterioles.

Thus, close to a 'closed' end the incident and reflected pressure waves are nearly in phase and sum together. Flow, on the other hand, is small so that the impedance is high. At a quarter-wave-length distance the incident and reflected waves are 180° out of phase and tend to cancel each other out, so that pressure oscillation is at a minimum while flow is at a maximum. The input impedance is, therefore, at a minimum. Maxima and minima thereafter succeed each other at quarter-wave-length intervals. With a system of fixed anatomical length the wave-length is dependent on the frequency of the oscillation, so that the first minimum to be found can be used, if the wave velocity is known, to define the distance at which the main reflexions are occurring. Because the waves are damped in travel the nearer we get to the origin the larger are the incident waves and the smaller the reflected waves, so that successive cancellation and summation of the waves produce smaller fluctuations.

A demonstration of these effects was made on a simple model consisting of two rubber tubes. The proximal tube connected to the pump was 120 cm long and had a bore of 0·6 cm; its wave velocity was *ca.* 1440 cm/sec. Into its end was fitted a thicker-walled tube of 0·35 cm bore which had a wave velocity of 2230 cm/sec. The distal tube was completely closed at its far end but was 7·5 m long to minimize the effect of reflexions from that end. The modulus of the input impedance measured in it is illustrated in Fig. 6. It can be seen from the values given that the proximal tube was one wave-length long at *ca.* 12 c/s and at 3 c/s there is a minimum in the amplitude of the impedance representing the quarter wave-length condition. There follows a maximum at 6 c/s (half wave-length) and another minimum at 9 c/s (three-quarter wave-length). This is very similar to the behaviour of the input impedance in the pulmonary bed, except that there is a much bigger variation between the maximum and the minima in the model because it has a much higher reflexion coefficient (see p. 439).

Similar considerations affect the phase shift of the wave and hence the apparent phase velocity. This is too complex to consider here, but we may briefly distinguish the true phase velocity, $c_0$, in a very long elastic tube which is solely determined by the elastic properties of the wall and assumes that the fluid has no viscosity. When filled with a viscous fluid we have the complex wave velocity, $c$, but except for very small vessels this may be taken as close to $c_0$. Finally, in the presence of reflexions we have the apparent phase velocity, $c'$, which is in excess of $c$ and $c_0$ in regions where the impedance is high and is below the true value where the impedance is low. This is only true for the 'spot' velocity. If the velocity is measured over a long interval the variations in apparent phase velocity are averaged out and a value approximating to the true velocity is found. For this reason the values of velocity for frequencies representing approximately a half wave-length and longer are chosen as representative of the true velocity, $c_0$. This averaging of phase velocities over the whole system was verified (but not illustrated) in the model described above. The phase velocity of these higher frequencies also approximates most closely to the 'foot-to-foot' velocity of a compound wave, such as that of the natural pulse wave (McDonald & Taylor, 1959).

## RESULTS

The input impedance was measured in 52 experimental runs on 24 animals. In addition, in 15 runs on 10 of these animals a second pressure record was made in a peripheral artery from which the wave velocity could be measured. Although, chronologically, these wave-velocity measurements were among the later experiments it is simpler and more logical to consider these results first.

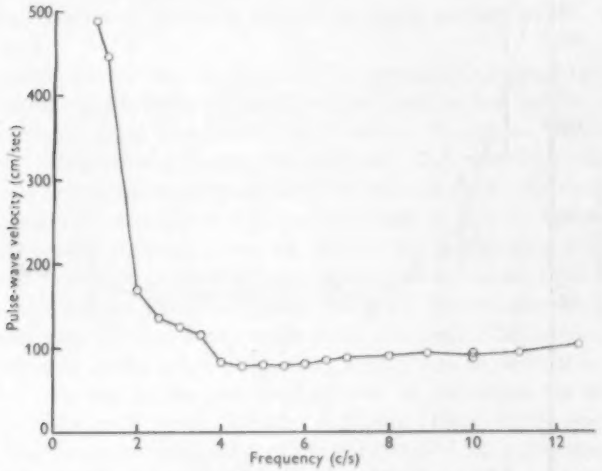



Fig. 2. The variation in apparent phase velocity in the pulmonary arterial tree with frequency in a single experiment. In this experiment the distal catheter was in the right lower lobe and its tip was 3·7 cm from the pulmonary valves.

### *Wave velocity in the pulmonary arterial bed*

The apparent phase velocity derived from a single experiment is shown in Fig. 2. It will be seen that it has a value of 500 cm/sec at 1 c/s, but this value falls steeply with increasing frequency until it reaches a value of 85 cm/sec at 4 c/s and thereafter remains relatively stable. There is a slight rise above 10 c/s and the velocity at 12·5 c/s is 105 cm/sec. In this experiment the catheter was in the right lower lobe and the interval between it and the pulmonary valves was 3·7 cm.

As explained under 'Interpretation of results' in Methods (p. 432), the high apparent phase velocities at low frequencies are due to the presence of reflexions occurring at less than one quarter wave-length distance and so give no measure of the true wave velocity. At higher frequencies, however, the interval is a considerable fraction of a wave-length and the effect of

reflexions is averaged out. The flat part of the curve is, therefore, taken as the characteristic wave velocity of the system over the interval measured.

A summary of all results of the 15 experiments is shown in Fig. 3, which displays the average value together with the S.E. of the mean. Results are also averaged for mean values of the frequency, e.g. the values at 4 c/s are for all readings in the range 3·6–4·5 c/s. The intervals over which these were measured ranged from 3·2 cm (when the catheter was in the middle lobe) to 7·0 cm (when in the tips of the upper or lower lobes). The mean value of

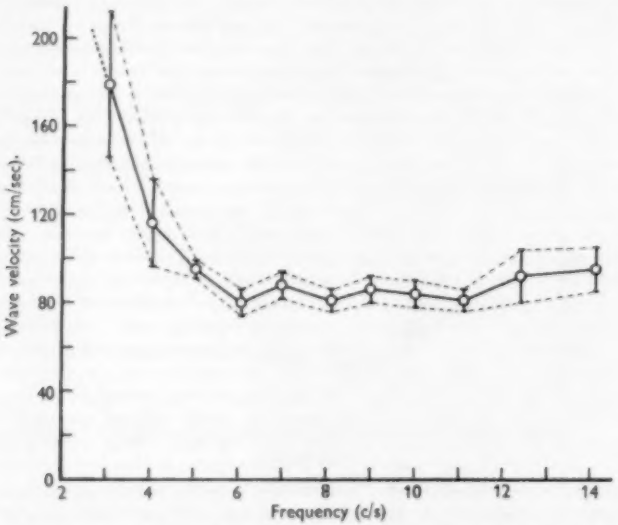



Fig. 3. Graph summarizing all measurements of wave velocity in 15 experiments. The mean values are shown by the open circles and full line; vertical bars show S.E. of mean. Each point averages all values at a mean frequency over a range of 1 c/s, e.g. the point at 4 c/s includes all results between 3·6 and 4·5 c/s. The mean interval between measuring points was 4·3 cm (range 3·2–7·0 cm). The mean wave velocity between 6·0 and 11·1 c/s was 83·3 cm/sec ± 5·7 S.E.

the interval was 4·3 cm. In the whole series it will be seen that a relatively stable value is not reached until 6 c/s and at low frequencies the apparent velocity is much higher. The mean value for the frequency range 6–11·1 c/s is 83·3 cm/sec ± 5·7 S.E. From 11·1 to 14 c/s the mean value is 93·3 cm/sec ± 11·1 S.E.

Velocities measured in arteries in the upper and lower lobes tended to be higher than those measured in arteries in the middle lobes, where the path length was shorter. In the latter, stable values were not reached until a rather higher frequency and this is attributed to the fact that a short

interval did not exceed a quarter wave-length until these higher frequencies were reached. This is the main reason for the large scatter at 3 and 4 c/s in Fig. 3 for the values of all experiments over widely differing intervals.

The lower wave velocity observed in the artery to the middle lobe is probably due to the wave velocity being lowest in the main pulmonary artery and becoming progressively higher as the arteries subdivide (see below). As the wave velocity measured over the whole interval is a weighted mean of the velocities in each successive segment, the relatively shorter second- and third-order arteries to the middle lobe will not increase the mean velocity as much as the longer small arteries in the upper and lower lobes.

Some support for this suggestion was given by measurements of the phase velocity made after the catheter had been pulled out for successive short intervals from the pulmonary valves. No great weight can be attached to these results because the accuracy of measuring small intervals in an easily extensible organ such as the lung is not great, and the technique was only used in one animal. The values obtained in this experiment were as follows: with interval 2 cm, i.e. within the pulmonary trunk, mean velocity 55·1 cm/sec; interval 3·2 cm, velocity 61·6 cm/sec; interval 4·2 cm, velocity 66·3 cm/sec; interval 5·5 cm, velocity 79·9 cm/sec. Velocities are mean values for the frequency range from 5 to 9 c/s. The volume flow was 3·3 ml./sec and as the main pulmonary artery was *ca.* 0·6 cm in diameter the linear velocity of the steady flow was *ca.* 12 cm/sec in this artery. From the findings of Patel, Schilder & Mallos (1960) in the dog it would appear that the cross-sectional area of the right and left pulmonary arteries together may be less than that of the trunk. Thus the correction for steady-flow velocity will be at least as high in these vessels. This suggests that the true value (see 'Sources of error' in Methods, p. 430) for wave velocity in the main pulmonary artery is about 45 cm/sec, or almost half that for the whole bed. As even over the full interval we were still only recording in arteries of 0·1–0·15 cm diameter, the flow-velocity correction may still be appreciable and all values recorded may be a little higher than the true wave velocity.

In one experiment the effect on wave velocity of altering the mean pulmonary artery pressure was tested. At 10 mm Hg the mean value of eleven observations in the frequency range 6–10 c/s was 87·6 cm/sec; with the arterial pressure at 20 mm Hg the mean value of the velocity (five observations) was 85·7 cm/sec. This suggested that pulmonary wave velocity did not vary significantly with mean pressure over the limits of what we presumed was the normal range, so that all other observations were made at 10 mm Hg.

The average wave velocity into all parts of the pulmonary arterial bed

may thus be taken as *ca.* 80 cm/sec. This enables us to predict the wave-lengths of oscillations that are imposed on it. Thus at 4 c/s the wave-length is 20 cm and a quarter wave-length will be 5 cm; at 8 c/s these distances will be halved, and so on proportionately. These dimensions are important in interpreting the behaviour of the input impedance.

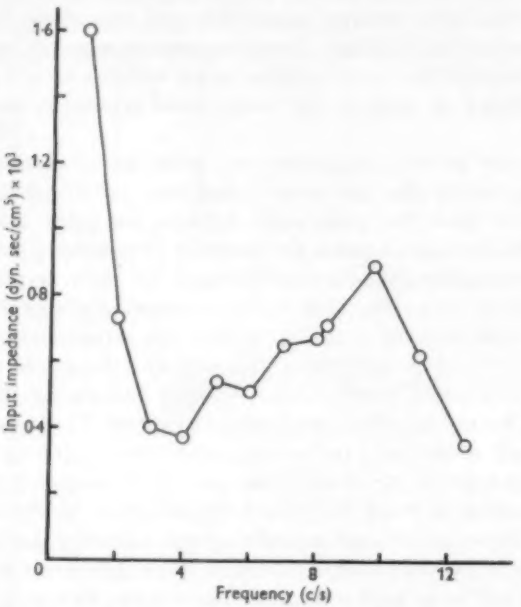



Fig. 4. The values of the modulus of the input impedance in a representative experiment, showing the variation with frequency. Rabbit, 3·1 kg.

*The input impedance of the pulmonary bed*

The results of a single experiment are illustrated in Fig. 4. It can be seen that the highest value of the modulus of the impedance is at 1 c/s, when it is $1{\cdot}6 \times 10^3$ dyn.sec/cm$^5$; with increasing frequency it falls to a minimum value, at 4 c/s, of 0·37 (the true minimum is probably slightly below 4 c/s). Thereafter it rises to another peak value, at 9·5 c/s, of 0·89 and then decreases to 0·35 at 12·5 c/s. From other experiments it can be predicted that this is close to another minimum and that the values would rise with increasing frequency. If the value at 12·5 c/s was a minimum, it can be seen that the peak value at 9·5 occurred at rather more than twice the frequency of the first minimum and that the second minimum fell at a little over three times this frequency. This suggested that the minima were

falling at frequencies representing the quarter and three-quarter wave-length situation and the maximum at a half wave-length for the system.

A graph summarizing the mean results of 52 experimental runs is shown in Fig. 5, together with the S.E. of the means. As in Fig. 3, the values have been averaged for frequency, i.e. the value for 6 c/s includes all results between 5·6 and 6·5 c/s. Considering that there has been no weighting for differences in size of animals or variations in resistance the scatter up to

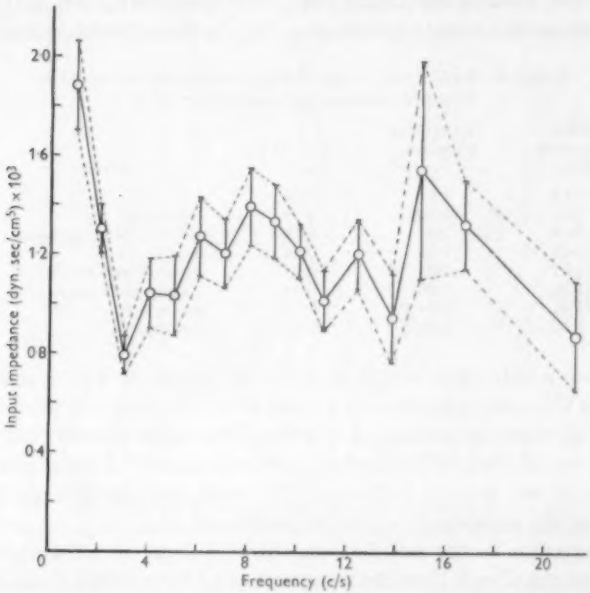



Fig. 5. Graph summarizing all measurements of pulmonary input impedance in 52 experiments on 24 animals. As in Fig. 3 the results have been grouped according to frequency at 1 c/s intervals and the mean frequency taken for each group. Average values of the input impedance are given by the open circles and solid line; vertical bars show the S.E. of mean.

15 c/s is remarkably small. The larger scatter in the higher frequencies may be partly attributed to the fact that fewer experiments were done in this frequency range, and they include a number of results that later standards would have rejected because the oscillatory pressure exceeded the mean pressure (see 'Sources of error' in Methods, p. 430).

From the whole set a similar pattern to that seen in Fig. 4 emerges. The impedance is high at low frequencies and falls to a minimum at 3 c/s. It rises again to a maximum at 8 c/s, falls to another minimum at 13·8 c/s, rises to another maximum at 15 c/s and thereafter decreases up to 21 c/s.

The precise values for the main points of possible interest are given in Table 1.

If the value close to 3 c/s is a minimum in input impedance such as occurs at a quarter wave-length in a system with reflexions then we can derive an estimate of the average length of the system. Taking the average wave velocity of 83·3 cm/sec then a quarter wave-length at this frequency is 6·96 cm. This may be compared with the value of 6 cm that Engelberg & DuBois (1959) took as the average length of the arterial bed in the rabbit lung. If this were a simple system (e.g. Fig. 7) there would be a maximum

TABLE 1. Some mean values of input impedance measured in 52 experiments on perfused rabbit lungs

| Mean frequency (c/s) | Impedance (dyn.sec. $cm^{-5} \times 10^3$) | S.E. of mean | Comment |
|---|---|---|---|
| 1·14 | 1·88 | 0·18 | — |
| 2·99 | 0·79 | 0·08 | minimum |
| 6·05 | 1·27 | 0·16 | ? subsidiary maximum |
| 8·19 | 1·39 | 0·16 | maximum |
| 11·11 | 1·01 | 0·12 | ? subsidiary minimum |
| 12·39 | 1·20 | 0·14 | ? subsidiary maximum |
| 13·81 | 0·94 | 0·18 | minimum |
| 14·87 | 1·54 | 0·44 | maximum |

representing a half wave-length at 6 c/s. Reference to Fig. 5 and Table 1 shows that there is a hint of such a peak here, but that it is overshadowed by the maximum occurring at 8·2 c/s. The three-quarter wave-length frequency would then be 9 c/s, where a minimum should occur (the nearest suggestion of one is at 11·1 c/s) and the whole wave-length maximum at 12 c/s (possibly suggested by the fluctuation at 12·4 c/s).

The maximum in the middle range is at 8·2 c/s, and if this is a half-wave-length maximum then the minimum at 13·8 c/s would fit fairly well as its corresponding three-quarter-wave-length value. This would correspond to a system length of 5·1 cm. This is also quite a reasonable figure of distance to the arterioles compared with the mean distance of 4·3 cm to arteries of 1·0–1·5 mm diameter recorded in the experiments on wave velocity. It is possible that reflexions from the closer arteriolar beds become dominant at the higher frequencies because, owing to the shorter length of path, damping of the waves in travel is less marked. The fact that the wave velocity tends to rise appreciably with frequencies over 10 c/s would also cause the wave-length to increase and so prevent the maxima and minima falling at exact multiples of the quarter-wave-length frequency.

The main conclusion is that the fluctuations of input impedance are due to reflexions. Considering the great range of distances to the peripheral arterial bed from the pulmonary trunk that is present in the lung it is more surprising that a definite pattern can be detected rather than the fact that

maxima and minima do not occur with the exact regularity of a simple model. The type of oscillations seen in such a model is shown in Fig. 6. It consisted of a length of rubber tube terminating in another tube of smaller bore and stiffer walls. The reflexion coefficient was estimated by means of Womersley's (1957) formula and estimated at 0·65. This is much higher than our results suggest for the pulmonary bed (rough calculations indicate a figure of *ca.* 0·25) so that the fluctuations in the amplitude of the impedance in the model are far greater than in the pulmonary bed. The similarities between the two systems are, however, quite easy to recognize.

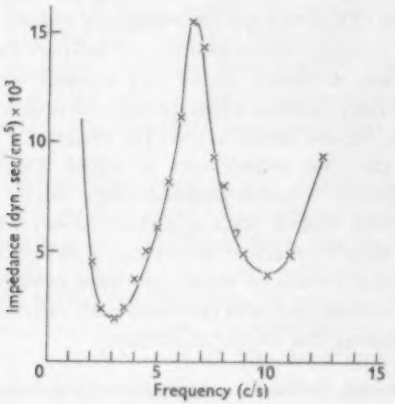



Fig. 6. The behaviour of the input impedance in a simple model with a single reflecting site. This shows marked similarities to the pattern seen in the pulmonary vascular bed (e.g. Fig. 4).

We do not present detailed results of measurements of the phase of the complex fluid impedance because a considerable diversity of pattern was seen in different experiments, and it does not appear that any useful information can be gained from its analysis. In a model with a single reflecting site there is a phase lead at low frequencies which falls to a value close to zero at the quarter-wave-length frequency, i.e. at the minimum of impedance amplitude. Following this the phase lags progressively to a point midway between the minimum and maximum, and returns to zero at the frequency of the next amplitude maximum. It then reverses once more and becomes a phase lead, reverses again at the three-quarter-wave-length frequency, and so on. Minor oscillations of this sort were observed in many experiments but in others they were quite overshadowed by a progressive reduction of phase lead as the frequency increased. Interpretation of results is further vitiated by the fact that, as measurement of

the phase was not originally a matter of interest, insufficient care was taken to ensure that the signs of the Fourier components were accurately recorded.

*Relation of impedance to vascular resistance*

In a previous communication Bergel *et al.* (1960) said that there appeared to be no obvious connexion between the impedance values and those for the pulmonary vascular resistance. This was based on a superficial comparison of the scatter of these two measurements. The measured range of resistance was from 2·0 to 22·0 $\times 10^3$ dyn.sec/cm$^5$. As the mean pulmonary artery pressure was almost always 10 mm Hg this represented flow rates of *ca.* 5–0·5 ml./sec. Thus the lowest resistance values in this range alone may be taken as roughly corresponding to normal values, i.e. a flow of 300 ml./min (Dittmer & Grebe, 1959). All animals were used until there was marked pulmonary oedema when the flow became too small to measure, with any precision, on the meter in use. The resistance was then classed as 'very high'. In the first experiment in which Dextran was used the resistance immediately became immeasurably high, apparently due to embolization of small vessels with agglutinated erythrocytes, as tests on microscope slides showed marked clumping of red cells with the Dextran in use. More normal values of resistance were obtained in later experiments when the vascular bed was perfused with saline solution for several minutes before starting the Dextran infusion.

TABLE 2. Relationship between the amplitude of the input impedance and the vascular resistance of the pulmonary vascular bed at varying frequencies

| Frequency (c/s) | No. of expts. | Regression line ($\|Z\| = a + b(R)$) | Correlation coefficient ($r$) |
|---|---|---|---|
| 1·0 | 8 | $0{\cdot}483 + 0{\cdot}137R$ | 0·97 |
| 2·0 | 12 | $0{\cdot}430 + 0{\cdot}060R$ | 0·94 |
| 3·0 | 12 | $0{\cdot}541 + 0{\cdot}018R$ | 0·59 |
| 4·0 | 12 | $0{\cdot}652 + 0{\cdot}0001R$ | 0·001 |

The interesting value of impedance to compare with the vascular resistance would be the impedance of the 'resistance' vessels. In so far as we consider the bed as having a partially closed termination, the impedance of these vessels is a terminal impedance. The closest approximation we can make to measuring this is by studying it at low frequencies when the length of the system, in terms of wave-length, is shortest. The correlation between the input impedance and the resistance in all experiments in which measurements were made at 2 c/s or below, and in which the resistance was recordable, is given in Table 2.

It can be seen that a satisfactory degree of fit for the regression lines is only found at 1 and 2 c/s. It will be seen, however, that the slopes show

that the rise in impedance is much less than the corresponding rise in resistance. This implies that the behaviour of some of the resistance vessels does not influence the terminal impedance significantly.

At 1·0 c/s the length of the system was about 1/16 of a wave-length as the impedance minimum fell at 4 c/s (1/4 wave-length) in this selected series. The highest ratio of impedance to resistance at this frequency was 1·13 : 2·13 or 53 %. The average value for the 5 experiments where the resistance was reasonable—below $4 \times 10^3$ dyn.sec.cm$^{-5}$—was 0·98 : 3·23 or 30 %. The ratio at the highest resistance recorded was 3·7 : 22·0 or 17 %. This illustrates the observation that impedance does not vary directly with resistance. Even so, all these values are greater than the corresponding ratio in the femoral bed, which is about 10 % (McDonald, 1960, Ch. 10). As the impedance increases very rapidly with reduction of frequency below 3 c/s it may well be that a value of 53 % at 1 c/s, for example, may represent a true terminal impedance that is quite close to the vascular resistance.

It should be emphasized that most of the changes in resistance we recorded were due to the gradual onset of pulmonary oedema, so that no physiological inferences can be made from these results. The preparation we used was not suitable for the investigation of vasomotor activity. In one experiment we did infuse noradrenaline (a total of 0·5 mg in a total flow in the run of 50 ml.) which caused a rise in the input impedance at 2·8 c/s from 0·6 to $0{\cdot}97 \times 10^3$ dyn.sec/cm$^5$. The resistance, unfortunately, was very high and could not be recorded. No effect on imput impedance could be detected with infusions of acetylcholine.

The virtually complete lack of any relation between impedance and resistance at 3 and 4 c/s emphasizes that in a system with partial reflexions and viscous damping there is no simple relation between its input impedance and the behaviour of its termination. In a simple system an increase in the terminal impedance will cause a reduction in the impedance at the quarter-wave-length minimum so that one would expect a negative slope to a regression line. However, as Table 2 shows, no significant regression line can be drawn from the values we have recorded in the pulmonary bed.

### *Amplitude of pressure in peripheral pulmonary arteries*

In the course of measuring the wave velocity in the arterial bed records were naturally also made of the amplitude of the pressure. Averaged over the whole frequency range the amplitude of oscillation was slightly higher at the periphery than at the origin, but the difference was not statistically significant. Around the frequency of the impedance minimum, however, the amplitude at the origin falls and becomes appreciably less than that in

the periphery (Fig. 7). For this reason we should expect a certain amount of peaking of the fundamental component of the naturally formed pulse wave. The 'peaking' observed is not, however, as marked as in the systemic system. It may be noted that the peripheral pressure was measured with the 'end' of the catheter and would, therefore, tend to be higher than a lateral pressure record because of the additional kinetic energy. With the low flow velocities that we may expect in such small vessels it is unlikely that the kinetic correction would be measurable.

*Oscillatory pressure in pulmonary veins*

Pulsatile venous pressure was recorded in lobar veins through a nylon catheter passed retrogradely from the left atrium. Over the frequency range 3–5 c/s the mean of 7 experiments was 0·62 cm $H_2O \pm 0{\cdot}12$ S.E. In the same experiments the average amplitude at the cannula was 2·52 cm $H_2O \pm 0{\cdot}32$ S.E. Thus the venous oscillation was rather less than 25% of that at the pulmonary valves. However, in many of these experiments resistance was considerably above what might be regarded as normal. It was found that the venous oscillatory pressure varied inversely with the vascular resistance, so that the normal ratio of venous to arterial pressure must be considerably greater than this.

A more interesting estimate of the amount of the arterial oscillation that passed through the capillary bed was given by comparing the oscillation in the peripheral arteries with that in the veins. The results of 6 experiments are shown graphically in Fig. 7, where the pressure oscillations at the origin of the pulmonary artery, in a peripheral artery and in a lobar vein, are compared. The average values at these three sites at the frequency of the impedance minimum were 1·56, 2·05 and 0·42 cm $H_2O$ respectively. In the case with the lowest resistance ($2{\cdot}2 \times 10^3$) the values were 1·55, 2·0 and 0·90 cm $H_2O$. The pulsation in the vein was then 58% of that at the origin and 45% of that in the peripheral arterial bed.

In some early experiments the flow from the atrial cannula appeared to be pulsatile at low frequencies but we thought it probable that this was due to vibration from the pump. No pressure oscillation was detected with the catheter tip in the atrium.

As was stated in Methods, no accurate measurements of wave velocity were possible from artery to vein as the length of the pathway was unknown. When we assumed a distance of double the interval between the origin and a peripheral artery the value obtained was about half that recorded in the arteries, i.e. 30–40 cm/sec. While a reduction of wave velocity during passage through the very narrow resistance vessels is to be expected (McDonald, 1960, Ch. 9) this evidence merely hints that this viscous effect is operating. The presence of this factor is supported by the

fact that the velocity tended to be lowest at low frequencies and climbed to a steady value with increase of frequency. It thus contrasted with the behaviour of the arterial velocity, which was very high at low frequencies (Figs. 3 and 4). The artery–vein velocity pattern is similar to that in a tube where the viscous effects are marked and reflexion is negligible. The main practical value of calculating these approximate velocities to the venous bed was to ensure that the phase shifts observed were consistent with a definite transmission time and that the apparent venous pressure recorded could not have been due to vibrations of the manometer transmitted from the pump or the direct effect of pulsations in contiguous arteries.

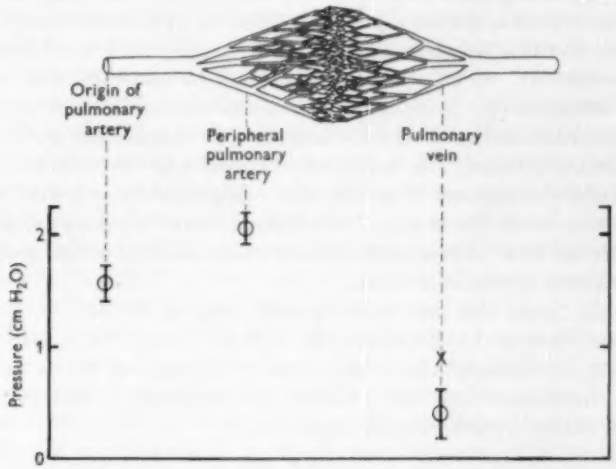



Fig. 7. A comparison of the amplitude of the oscillatory pressure in various regions of the pulmonary vascular tree. The results are for 6 experiments in which this was measured at three sites. Results are shown for the frequency of the first impedance minimum (3–4 c/s), i.e. when the oscillatory pressure at the origin was least in comparison with that in the peripheral arteries. At double this frequency when impedance is at a maximum the oscillatory pressure at the origin is rather greater than that in the periphery. Mean values are shown by circles with a vertical bar to show the S.E. of mean. The venous pressure oscillation in the case with the lowest resistance ($2{\cdot}2 \times 10^9$ dyn.sec/$cm_5$) is shown by a cross—the corresponding arterial pressures fell on the mean values and are not shown.

## DISCUSSION

When studying the behaviour of pulsatile flow in a vascular bed it is insufficient to record the form of the pressure wave, although this is the easiest measurement to make. Even if the flow pattern is also measured, or derived indirectly, comparisons of the wave forms of pressure and flow

can only be made descriptively (e.g. Baxter & Pearce, 1951, described waves as 'rounded', 'round-triangular' or 'triangular') unless some form of mathematical analysis is used to extract quantitative data. The method we use is that of describing a pulse by a Fourier series, i.e. a set of harmonic oscillatory components at frequencies which are integral multiples of the pulse frequency. At a single frequency the input fluid impedance is then the ratio of the oscillatory pressure at the origin to the oscillatory flow. This method has been used fruitfully in the systemic arterial bed (McDonald & Taylor, 1959; McDonald, 1960). A greater range of frequencies may be studied by the technique used in the present work where a sinusoidal flow from a pump is imposed on a steady flow through perfused lung. The input impedance of such a system is greatly influenced by the wave-length of the oscillation and this is determined by the frequency (which is known) and the wave velocity. It was therefore necessary to determine this velocity.

*Pulse-wave velocity.* In contrast to the literature on the systemic arteries there appear to have been very few attempts to measure the wave velocity in pulmonary arteries. The value we obtained was 83·3 cm/sec $\pm$ 5·7 S.E. At first sight this appears to be very low, being only about 1/5 of the value of *ca.* 400 cm/sec in the aorta. Intuitively, however, it does not seem unreasonable in view of the much greater distensibility of the pulmonary compared with systemic arteries.

The only figure this can be compared with, in the rabbit, is that of 200 cm/sec derived by Engelberg & DuBois (1959). They assessed the velocity, $c_0$, by means of the modification of the classical Moens–Korteweg formula introduced into this country by Bramwell & Hill (1922), but proposed earlier by Otto Frank, which is:

$$c_0 = \sqrt{\left(\frac{V.\delta P}{P.\delta V}\right)}. \tag{9}$$

The volume distensibility or elastance, $\delta P/\delta V$, was measured for the whole arterial bed after the capillaries had been blocked by emboli (it was quoted as its reciprocal, the compliance). The total volume, $V$, however, had to be derived by the assumption of a simple geometrical relation for the change of volume at each subdivision of the arteries. Furthermore, the equation used is for a single thin-walled elastic cylindrical tube, filled with a non-viscous liquid. It is doubtful whether it can be regarded as more than an approximate solution for a rapidly subdividing system of tubes filled with a viscous liquid. The comparison of their derived value and our measured value implies that there are marked errors (making $V$/compliance some four to five times too large) in their figures.

The reported estimates of wave velocity in the pulmonary bed in other species are also higher than those we recorded. Patel *et al.* (1960) measured

the ratio of the changes in radius and pressure ($\delta R/\delta P$) in the pulmonary artery of the dog and the velocity derived from these we calculate as about 250 cm/sec for their average data. The mean pressure was much higher (22 cm $H_2O$) for their experiments which may account for some of this difference, and there is also a species difference. On the other hand, our results imply that the velocity for the whole bed would be considerably higher than that for the pulmonary trunk. A much earlier measurement by Johnson, Hamilton, Katz & Weinstein (1937) gave a value of 400 cm/sec. The implications of these values are discussed below in regard to speculation on the behaviour of the input impedance.

In man, Fleischner, Romano & Luisada (1948) estimated the main pulmonary arterial wave velocity as 200 cm/sec and the more peripheral arteries to have a velocity of 275 cm/sec. These measurements were made by kymographic techniques which measure delay in dilatation between the pulmonary knob and the right hilar shadow, and between the right hilar shadow and the visible base of the right lung. In view of the possible errors in measuring the precise dimensions of X-ray shadows these figures must be regarded with caution. As recording in the pulmonary artery with a double-lumen catheter is standard practice in many clinics, the taking of suitable pressure records, a known distance apart, should make precise measurements of foot-to-foot wave velocity a relatively easy matter—although none appear to have been made as yet.

Following Engelberg & DuBois (1959), Milnor, Jose & McGaff (1960) have endeavoured to measure the distensibility of the total pulmonary vascular bed in terms of compliance. Their estimation of true pulmonary vascular volume in man is very elegant but it is difficult to see how they can estimate compliance on a single measurement of volume against pressure. Distensibility must be defined as a ratio of the change of volume to the change of pressure. For the lung, which is a closed bag (albeit complicated geometrically) this is a useful measurement. For the vascular bed, which is also geometrically complex but in addition has a large leak at the far end, it would seem very difficult to separate distensibility from flow. In addition, the only values that Milnor *et al.* (1960) can derive are for the whole pulmonary vascular bed, whereas by far the greatest part of the pulsatile flow is on the arterial side of the capillaries. It would appear to us that the elastic properties of the pulmonary arterial bed would be much more simply characterized by measurements of arterial wave velocity. The techniques of studying lung mechanics are not easily applied to those of a vascular bed where both through flow and change of volume are taking place.

*Input impedance*

In the presentation of Results it has been seen that the amplitude of the input impedance is highest at very low frequencies and falls to a minimum value at 3–4 c/s. Thereafter it rises to a maximum value of about 8 c/s and subsequently falls and rises again with increasing frequency. This behaviour is typical of a fluid-filled elastic system in which reflexions are occurring and this is the interpretation we have put on them. It is indeed difficult to see any other way in which this behaviour could be explained.

Reflexion of waves propagated in an elastic tube will occur at any point where the fluid impedance changes. It was shown by Womersley (1957) that at any subdivision into branches one cannot achieve exact matching of the complex impedances on either side of the junction. At branches of the larger vessels, however, he predicted that the discrepancy would be small. Theoretically it is much greater at the subdivision of vessels of the magnitude of the smallest arteries, and experimental evidence in systemic vascular beds indicates that the main reflecting sites are in the region of the arteriolar beds (McDonald, 1960). The value for the average length of the arterial bed derived from our Results lies between 5 and 7 cm, which when compared with the anatomical size of the rabbit lung suggests that in this vascular bed the main site of reflexion is in the minute vessels. This estimate agrees very reasonably with that of 6 cm made by Engelberg & DuBois (1959).

The only other estimate of the impedance of the pulmonary arterial system is also by these last authors. This was derived by computing the behaviour of a simple analogous electrical circuit. It was an oscillatory circuit consisting of an inductance and capacitance, with a resistance connected in parallel across the condenser. The inductance represented the mass of the blood and its value was based on an estimate assuming a certain geometrical arrangement of subdivision of the pulmonary arteries. The capacitance represented the compliance which had been measured. The resistance represented the total vascular resistance of the bed and was regarded as residing principally in the smallest vessels such as the capillaries. Apart from the necessary approximations involved in deriving the dimensions of these components, especially the inductance, the authors regarded the omission of any transmission time effect as being a permissible, first-order approximation. At first sight this may seem reasonable for, taking their assumed wave velocity of 200 cm/sec, a 6 cm system is only 1/10 wave-length at the average pulse frequency they found (200 c/min). In fact this is far from negligible, for at the frequency of the second harmonic the system is 1/5 wave-length which is close to the relative node

at 1/4 wave-length. For the fifth harmonic it is a half wave-length, which by no approximation can be regarded as very short. In fact they calculated the behaviour of their analogue up to $10^4$ c/min when the system is more than 5·5 wave-lengths.

From our data the system is about 1/4 wave-length at 3 c/s (180 c/min) so that the approximation is even less valid. Nevertheless it is interesting to compare their graph with our own (Fig. 8). They found a minimum of impedance at 4·3 c/s (260 c/min), which is not far removed from many of

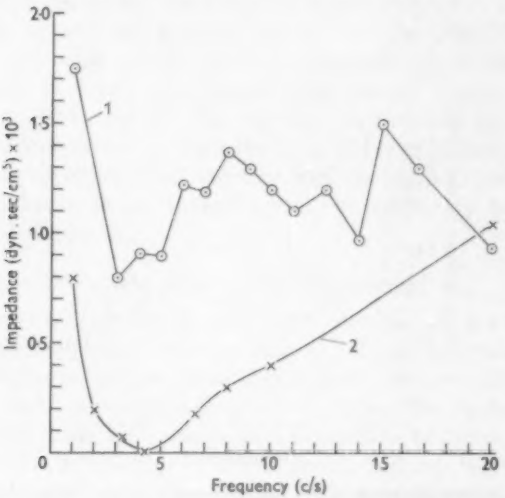



Fig. 8. A comparison between the values of the fluid input impedance in our experiments and those calculated from an electrical analogue by Engelberg & DuBois (1959), and described in the text. Curve 1 (⊙) shows the mean values of all our experiments and is taken from Fig. 5. Curve 2 (×) is a transcription of a part of their graph—it is only approximate, for this graph is small in scale and plotted with a logarithmic abscissa for frequency.

our observed values (e.g. Fig. 4) although rather higher than our over-all average. There is, however, a big discrepancy in the value of the impedance at this minimum for they calculated it to be 8 dyn.sec/cm$^5$ whereas we found the mean to be 800 dyn.sec/cm$^5$. At lower frequencies the values in both curves rise sharply but at 1c/s it is only about $0{\cdot}8 \times 10^3$ in their graph, whereas our mean is over $1{\cdot}8 \times 10^3$. With increasing frequencies above the minimum their graph rises steadily and is quite unlike the curve we find, and up to 20 c/s is always below the values we obtained. It is not felt, therefore, that this rather involved method of deriving the impedance by the use of this analogue circuit is very useful. Even with a circuit in the

form of a transmission line (Taylor, 1957*a*) it has been shown that it cannot fully mimic a fluid-filled system (Taylor, 1959).

Clearly the use of perfusion and an oscillatory pump is of no value in a living animal. If the flow and pressure in the pulmonary artery can be measured the input impedance at the frequencies represented by the harmonic components of these pulses may be determined. As an example we have taken some curves recorded in the cat by Baxter & Pearce (1951).

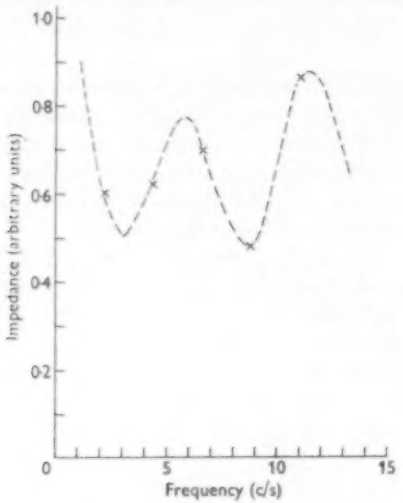



*Fig.* 9. The input impedance in the cat pulmonary artery derived from simultaneous records of pressure and flow by Baxter & Pearce (1951, Fig. 10). The pulse frequency was 2·2 c/s and the values for the first five harmonic components have been calculated by the use of Fourier analysis. These values are shown by crosses. The dotted line shows an impedance variation with frequency similar to that found in the rabbit and it can be seen that these points might well fall on such a curve. The phases of the harmonic components add support to this view.

These were enlarged and subjected to Fourier analysis. The modulus of the impedance for the first five harmonics in one pair of curves is shown in Fig. 9. As the fundamental frequency was 2·2 c/s the available points are insufficient to delineate the curve with the precision that we could using the pump (Fig. 4), but the line drawn through them based on the rabbit data shows they might well be points on such a curve. That is, the first two harmonics (2·2 and 4·4 c/s) probably fall on either side of a minimum at *ca.* 3 c/s followed by a maximum at 6 c/s and another minimum at 9 c/s with a subsequent rise at 11 c/s. As the size of the lung in the cat is much the same as that of the rabbit, if this is a valid comparison it would imply

that the pulmonary wave velocity is about the same value in both species. It is unfortunately not possible to compare the value of the impedance from Baxter & Pearce's curves with our own, as theirs were not calibrated.

This technique of measuring the input impedance could be employed in man, using the indirect method that Bergel, McDonald & Taylor (1958) used in the dog femoral artery. With the use of a double-lumen catheter with two lateral apertures sufficiently close together, so that they both lay in the main pulmonary artery, the velocity of the pulsatile flow could be derived from the pressure gradient while the pressure in the pulmonary artery could be recorded through one of the channels. Thus with the addition of very little complexity to routine recordings made in cardiac catheterization in many laboratories considerably greater quantitative information could be gained. For whereas the shape of the pressure and flow pulses is determined by both the form of the systolic ejection by the heart and the properties of the vascular bed, the input impedance is determined by the latter alone. How much of the pulmonary bed is involved remains to be discussed.

### *The extent of the pulmonary 'arterial' bed*

Properly speaking the input impedance involves all the system up to the point where oscillatory flow is damped out. As we recorded pressure oscillations in the pulmonary veins this is more extensive than the arterial bed, but this last plays such a dominant part that for the sake of brevity that region determining the input impedance may be called 'arterial'.

As this system terminates in the capillary bed one might expect the terminal impedance to change with the resistance of the bed. As is seen from Results (p. 441), there is a rise in the input impedance at 1 c/s (which is the nearest we were able to get to measuring the terminal impedance) and the resistance. Also, the impedance at this frequency was much closer to the resistance in the pulmonary bed than corresponding values in the femoral bed. In the latter case this was interpreted by McDonald (1960) as indicating that only the proximal arterioles were included in the region which determined the impedance. Nevertheless, the 'terminal' impedance could be increased two to three times by intra-arterial noradrenaline. In the lungs we have studied the changes in 'terminal' impedance were much less than this with equally large changes in the resistance. These changes in resistance, however, were not due to arteriolar constriction but to pulmonary oedema, which presumably exerts a far greater effect on the capillaries and venules than it does on the arterioles. The fact that the impedance does vary quite appreciably with the resistance indicates that the effective end of the arterial system is probably at the distal end of the arterioles or in the capillary bed.

Any reflexions of the oscillations that pass through the capillary bed must be very small because they will be heavily attenuated by that bed. One might expect that such as do occur would tend to be out of phase with the arterial reflexions, for a wave travelling towards the left atrium from the capillaries is moving towards an 'open' end in contrast to the partially closed end of the arterial system.

The length of the arterial system that we are interested in when concerned with oscillatory flow is in terms of wave-length. The main variables determining this are the frequency and the wave velocity. The actual anatomical length is by comparison relatively fixed; this will vary somewhat with respiration and with changing vasomotor activity the effective termination may shift slightly. These variations are very small compared with the range of frequencies represented in the cardiac pulse and probably small compared with changes that may occur in wave velocity, e.g. with alterations of mean pulmonary artery pressure or pathological changes affecting the stiffness of the arterial wall.

Changes in the length of the system, in this sense, will alter the frequency at which the input impedance is at a minimum. This means that the pressure necessary to create a given flow is least at this frequency. As the oscillatory load on the ventricle is then at its smallest it is interesting to note that the frequency for this minimum approximates to that of the normal pulse frequency. Thus the fundamental component of the pulse wave will normally be working against a low impedance and as this is always the largest component it may well be that this fact plays a part in determining the resting pulse rate. The situation is, however, more complex than this implies because the cardiac pulse is a compound wave containing a range of frequencies. Therefore an impedance minimum would be to some extent balanced out by the fact that the second harmonic would be working at an impedance maximum and this is also a component of considerable magnitude (in the example of Fig. 9 its amplitude was 70% of the fundamental). The values shown in Fig. 5 do indicate, however, that a marked fall in pulse frequency, say to 1 c/s, would cause a great increase in the load on the right ventricle. Any marked increase in stiffness of the pulmonary artery by shifting the minimum to a higher frequency would have a similar effect. Observations on larger species would be of interest in this respect, for a greater anatomical length may be offset by a faster wave velocity (as values quoted for the dog suggest), but in fact the resting pulse frequency is also less. Without more data it is difficult to assess the relative importance of these two factors.

It is possible that the main significance of the frequency-dependence of the input impedance of the pulmonary circulation is to be sought in the balance between right and left ventricles. One exploratory experiment on

the input impedance of the rabbit aorta suggests that the quarter-wavelength minimum is also about 3 c/s, a result which parallels observations in the dog (McDonald, 1960). This dynamic similarity between the systemic and pulmonary circulations is due to the fact that although the anatomical length of the latter is much shorter the wave velocity is correspondingly lower. Thus the impedances against which the right and left ventricles are working are matched and the optimal frequency will be the same for both.

### *Venous pulsation*

The finding of pulsation in the pulmonary veins was to be expected after the observation of Lee & DuBois (1955), using the 'body plethysmograph' technique, that flow in the pulmonary capillaries was pulsatile. It would be interesting to be able to estimate the damping in the capillary bed, but this would be far from precise from our results as the venous pressure was measured at some distance from the capillary bed. As noted above, flow from the capillaries on the venous side is travelling towards an 'open' end which will, in general, cause a reduction in the amplitude of the pressure wave. However, without knowing more about the physical characteristics of the venous bed it is impossible to predict how these changes will vary with frequency. The largest oscillation recorded in a lobar vein was some 45% of that recorded in a small artery in the same lung. It would be interesting to compare these findings with pressure records made with a 'wedged' arterial catheter and from the left atrium. Since the transmitted oscillation diminished as the vascular resistance increased it seems that damping in the small vessels is the major factor in determining the size of venous pulsation.


## SUMMARY

1. In perfused rabbit lungs an oscillatory flow has been imposed by a pump with a sinusoidal output. The pressure oscillations were measured at the origin of the pulmonary artery. The ratio of oscillatory pressure and flow was recorded as the input impedance.

2. In some experiments the oscillatory pressure was also recorded in a small artery in one of the lobes of the lung. From the phase shift between the two points the wave velocity was calculated. In 15 experiments the mean value was $83{\cdot}3 \pm 5{\cdot}7$ cm/sec at 6–11 c/s. At lower frequencies it rose markedly; this is attributed to the presence of reflected waves altering the apparent wave velocity.

3. The input impedance was also high at low frequencies and fell to a minimum at 3–4 c/s, where its mean value in all experiments was *ca.* 800 dyn.sec/cm$^5$. Thereafter followed a maximum at *ca.* 8 c/s and another minimum at 12–14 c/s.

4. This pattern is interpreted as due to reflexions arising from the termination of the arterial bed which would cause a minimum when the system is either one quarter or three quarters of a wave-length. The mean length of the arterial system at the wave velocity recorded lies between 5 and 7 cm, which fits reasonably with the anatomical dimensions.

5. The vascular resistance varied considerably in various experiments and the input impedance at 1 c/s varied with it but to a much smaller degree. The implications of this in regard to the main site of reflexions is discussed.

6. Oscillatory pressure in the veins was measured in 7 experiments. The transmission through the capillary bed varied inversely with the vascular resistance. In the lung with the lowest resistance ($2{\cdot}2 \times 10^3$ dyn.sec/cm$^5$) the pulsation in the lobar veins was 45 % of that in a small artery and 58 % of that at the pulmonary valves. These values are for the frequency of the first impedance minimum.

7. It is pointed out that for oscillatory flow the behaviour of the input impedance, which represents a considerable part of the load on the ventricle, is determined by the wave-length of the system, i.e. its wave velocity and the oscillatory frequency. As the pulmonary arterial system has a much lower wave velocity than the systemic arteries, their effective lengths are comparable in spite of the great disparity of their anatomical lengths. The significance of this in matching the optimal working conditions of the right and left ventricles and the part it may play in determining the normal pulse frequency is discussed briefly.

We are grateful to Professor W. Melville Arnott for giving C. G. C. leave of absence to carry out this work and to Professor M. de Burgh Daly for providing facilities. Dr D. H. Bergel gave invaluable help in setting up the apparatus and in performing many of the experiments. The pump and the harmonic resolver were built by Dr M. G. Taylor and for the gift of these and aid in the planning of this type of experiment we are deeply indebted to him. Some of the apparatus was purchased originally with a Grant-in-Aid from the Royal Society to M. G. T. and some purchased by the Medical Research Council for D. A. McD.

## REFERENCES

Baxter, I. G. & Pearce, J. W. (1951). Simultaneous measurement of pulmonary arterial flow and pressure using condenser manometers. *J. Physiol.* **115**, 410–429.

Bergel, D. H., Caro, C. G. & McDonald, D. A. (1960). The input impedance of the pulmonary vascular bed. *J. Physiol.* **154**, 18–19*P*.

Bergel, D. H., McDonald, D. A. & Taylor, M. G. (1958). A method for measuring arterial impedance using a differential manometer. *J. Physiol.* **141**, 17–18*P*.

Bramwell, J. C. & Hill, A. V. (1922). The velocity of the pulse-wave in man. *Proc. R. Soc.* B, **93**, 298–306.

Caro, C. G. & McDonald, D. A. (1960). Pulse-wave velocity in pulmonary vessels. *J. Physiol.* **153**, 68–69*P*.

Cotton, K. L. (1960). *The instantaneous measurement of blood flow and of vascular impedance.* Ph.D. Thesis. University of London.

DITTMER, D. S. & GREBE, R. M. (1959). *Handbook of Circulation*. Table 42, p. 80. Saunders: Philadelphia.

ENGELBERG, J. & DUBOIS, A. B. (1959). Mechanics of pulmonary circulation in isolated rabbit lungs. *Amer. J. Physiol.* **196**, 401–414.

FLEISCHNER, F. G., ROMANO, F. J. & LUISADA, A. A. (1948). Studies of fluoro-cardiography in normal subjects. *Proc. Soc. exp. Biol., N.Y.*, **67**, 535.

JOHNSON, V., HAMILTON, W. F., KATZ, L. N. & WEINSTEIN, W. (1937). Studies on the dynamics of the pulmonary circulation. *Amer. J. Physiol.* **120**, 624–634.

LEE, G. DE J. & DUBOIS, A. (1955). Pulmonary capillary flow in man. *J. clin. Invest.* **34**, 1380–1390.

MCDONALD, D. A. (1960). *Blood Flow in Arteries*. London: Arnold.

MCDONALD, D. A. & TAYLOR, M. G. (1959). The hydrodynamics of the arterial circulation. *Progr. Biophys.* **9**, 107–173.

MILNOR, W. R., JOSE, A. D. & MCGAFF, C. J. (1960). Pulmonary vascular volume, resistance, and compliance in man. *Circulation*, **22**, 130–137.

PATEL, D. J., SCHILDER, D. P. & MALLOS, A. J. (1960). Mechanical properties and dimensions of the major pulmonary arteries. *J. appl. Physiol.* **15**, 92–96.

TAYLOR, M. G. (1957*a*). An approach to an analysis of the arterial pulse-wave. I. Oscillations in an attenuating line. *Phys. Med. Biol.* **1**, 258–269.

TAYLOR, M. G. (1957*b*). An approach to an analysis of the arterial pulse-wave. II. Fluid oscillations in an elastic pipe. *Phys. Med. Biol.* **1**, 321–329.

TAYLOR, M. G. (1959). An experimental determination of the propagation of fluid oscillations in a tube with a visco-elastic wall; together with an analysis of the characteristics required in an electrical analogue. *Phys. Med. Biol.* **4**, 63–82.

WOMERSLEY, J. R. (1957). The mathematical analysis of the arterial circulation in a state of oscillatory motion. *Wright Air Development Center, Technical Report* WADC-TR 56-614.

*J. Physiol.* (1961), **157**, *pp.* 454–461
*With* 3 *text-figures*
*Printed in Great Britain*

# THE ACTION OF CHLOROTHIAZIDE IN THE PERFUSED CAT KIDNEY

BY T. DE LIMA AND MARY F. LOCKETT
*From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W. 3*

(*Received* 23 *January* 1961)

Crawford & Kennedy (1959) found the prolonged saluretic and diuretic effects which characterize the action of chlorothiazide in man (Laragh, 1958; Bayliss, Marrack, Perkis, Rees & Silva, 1958; and others) and in dogs (Beyer, 1958) were absent both in clinical and experimental diabetes insipidus. The first dose of chlorothiazide caused loss of salt without change in urine volume in these diabetics, so that the osmolarity of the urine rose. Thereafter the saluresis abated but the urine remained concentrated so long as the drug was given, and the daily volume of urine remained about halved. Kennedy & Crawford (1959) attributed this adaptation to the initial saluretic effects of chlorothiazide to change in the effects of the drug on tubular function. Then de los Reyes, Gómez & Bradford (1960) found that chlorothiazide decreased the filtration rate in diabetic patients; this they considered sufficient to explain the decrease in urine volume. They were confirmed in their observation by Dávid, Lázlo & Kovács (1960), who also showed that the antidiuretic action of chlorothiazide in patients with diabetes insipidus was absent in patients suffering from psychic polydypsia, although both groups of patients lack circulating anti-diuretic hormone (ADH).

Davey & Lockett (1960) found differences in the actions of aldosterone monacetate on the isolated cat kidney related to the presence or absence of a circulation through the head during the collection of blood for the perfusion circuit and the making of the heart-lung-kidney preparation. They attributed this difference to the absence of an oxytocin-like substance from blood collected in the absence of a head circulation. We were therefore interested to compare the actions of chlorothiazide on isolated kidneys perfused with these two types of blood, and to discover whether oxytocin modified the effects of chlorothiazide in these preparations.

## METHODS

Male, female or neuter cats were used. Anaesthesia was induced with ether and was maintained by the intravenous injection of 1·0 % chloralose in 0·9 % NaCl, 8·0 ml./kg. The larger cats were used as blood donors. Heparinized blood (heparin 1500 u./100 ml. blood)

was withdrawn by femoral arterial cannula, either without further interference with the circulation (intact donors) or after ligature of the brachiocephalic and left subclavian arteries and section of the vagi (headless donors). The blood was usually stored overnight at 7° C and next day until the heart-lung-kidney preparation was being made. Then 60–120 mg $NaHCO_3$ in 2–5 ml. 0·9 % NaCl was added. To each 100 ml. blood was added 3·0 ml. of a mixture of 5 % creatinine and 2·5 % *p*-aminohippuric acid (PAH) in 0·9 % NaCl, and an infusion of PAH was made into the venous reservoir to maintain a constant concentration of PAH in the blood.

Heart-lung-kidney preparations were made in cats weighing 1·1–1·8 kg as described by Davey & Lockett (1960), without exclusion of the head from the circulation until approximately 4–5 min before the perfusion of the kidney began. The temperature of the blood and the kidney chamber varied from 36·0 to 37·0° C and the arterial pressure from 114 to 148 mm Hg on different occasions, but these remained constant throughout each experiment.

The methods used for the measurement of renal blood flow (R.B.F.), the collection of urine and blood samples, the estimation of sodium, potassium and creatinine in blood and urine, have been reported (Davey & Lockett, 1960). The carbon dioxide combining power of plasma was estimated by the method of Hawk, Oser & Summerson (1947), and *p*-aminohippuric acid in plasma and urine by the method of Bratton & Marshall as modified by Smith, Finkelstein, Aliminosa, Crawford & Graber (1945).

Chlorothiazide (Merck, Sharp and Dohme Ltd), Pitocin and Pitressin (Parke Davis and Co. Ltd), creatinine, and inulin (British Drug Houses Ltd), *p*-aminohippuric acid (L. Light and Co. Ltd) and mannitol (Hopkin and Williams Ltd) were obtained directly from the manufacturers.

## RESULTS

### *The effect of chlorothiazide on cat kidneys perfused with blood drawn from animals in which the head had not been excluded from the circulation (intact donors)*

The influence of chlorothiazide on the excretion of sodium and potassium ions and water was examined at spontaneous rates of urine flow in four heart-lung-kidney preparations perfused with blood collected from intact donors. Figure 1 shows results from one experiment typical of this series. When the urine and blood flows had remained nearly constant for half an hour, 10 mg chlorothiazide was injected into the venous reservoir to mix with 145 ml. of circulating blood. Within 10 min the rates of urine flow and of excretion of Na and K had begun to increase and the $T_m$ of *p*-amino hippuric acid (PAH) to decrease. The maximum effects of chlorothiazide on urine flow, excretion of these ions and $T_m$ PAH were reached in 20–30 min, were well maintained, and were unaccompanied by changes in R.B.F. or creatinine clearance. The $CO_2$ combining power of the plasma fell steadily throughout all experiments, sometimes reaching values as low as 29 ml./100 ml. Neither this, nor the addition of 40 m-u. vasopressin/150 ml. circulating blood influenced the recorded actions of chlorothiazide.

*The effect of chlorothiazide on cat kidneys perfused with blood drawn from animals in which the head had been excluded from the circulation (headless donors)*

Kidneys perfused with blood taken from headless donors excreted a greater proportion of the filtered load of Na than those perfused with blood

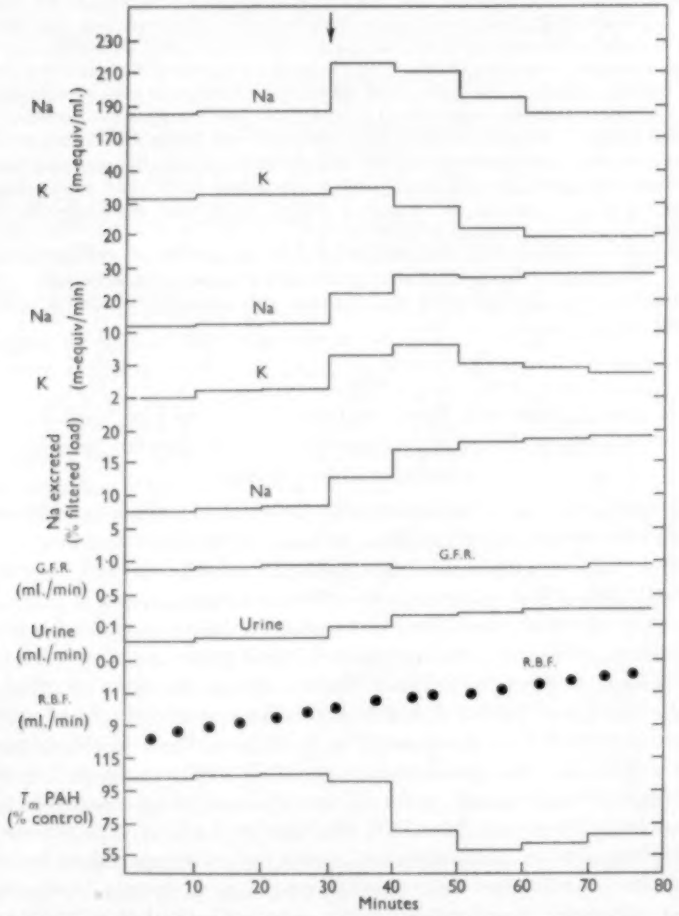



**Fig. 1.** The effect of chlorothiazide on the excretion of $Na^+$, $K^+$ and water by a cat's kidney, 7·4 g, perfused at 142 mm Hg with blood taken from an intact donor. Ordinates, from above downward: urinary concentration of Na, K; urinary excretion of Na, K; Na excreted (% filtered load); G.F.R.; urine volume; R.B.F.; $T_m$ PAH. Abscissae, time. Chlorothiazide, 10 mg/145 ml. blood given at arrow.

taken from intact animals (Table 1). The effect of chlorothiazide in blood from headless donors was also characteristic and is described by reference to a typical result in Fig. 2. A small increase in urine flow, accompanied by an increase in the rate of excretion of Na and K ions and decrease in $T_m$ PAH, became evident in the 10 min immediately following the addition of 10 mg of the drug to the venous reservoir. The concentrations of Na and K ions in the urine remained unchanged (Fig. 2) or rose slightly to reach a maximum in the next 10 min period. Thereafter these concentrations remained unaltered, but the creatinine clearance and urine volume decreased progressively and in parallel. The proportion of the filtered load of Na excreted was raised by chlorothiazide, then remained constant; it did not decline as the rates of filtration and excretion of Na and K decreased (Fig. 2).

TABLE 1. The effect of chlorothiazide (10 mg/140–150 ml.) on cat kidneys perfused with blood taken from intact animals is contrasted with the action of the drug in kidneys perfused with blood from headless donors

| | In blood from intact donors | | In blood from headless donors | |
|---|---|---|---|---|
| Effect measured | Before drug | At maximum effect of drug | Before drug | At maximum effect of drug |
| Urine flow (ml./min) | 0·063 ± 0·014 (4) | 0·116 ± 0·038 (4) | 0·20 ± 0·05 (4) | 0·15 ± 0·07 (4) |
| Na excretion rate ($\mu$-equiv/min) | 5·8 ± 2·56 (4) | 15·9 ± 2·89 (4) | 34·1 ± 4·1 (4) | 37·2 ± 3·8 (4) |
| Na excreted (% filtered load) | 3·6 ± 1·95 (4) | 9·7 ± 3·41 (4) | 11·7 ± 3·09 (4) | 21·9 ± 3·14 (4) |
| Creatinine clearance | 0·89 ± 0·04 (4) | 0·88 ± 0·02 (4) | 1·53 ± 0·21 (4) | 0·84 ± 0·19 (4) |
| Weight of kidneys (g) | 7·95 ± 0·63 (4) | | 9·84 ± 1·56 (4) | |

The values given are means ± standard error of the mean, followed by the number of preparations within brackets. Each kidney was perfused with one type of donor blood only.

Oxytocin, 20 m-u./140–150 ml. blood, produced only short-lasting antagonism of reductions in filtration rate caused by chlorothiazide in these preparations, and did not alter the proportions of the filtered load of sodium reabsorbed (Fig. 3).

## DISCUSSION

The first point of interest arising out of these experiments is the very great similarity of the effect of chlorothiazide in man (Laragh 1958; Bayliss *et al.* 1958) and in intact animals (Beyer, 1958) on the excretion of sodium, potassium and water, and the effect of this drug on isolated cat kidneys perfused with blood drawn from intact animals (Fig. 1). In these perfused preparations chlorothiazide decreased the reabsorption of sodium and water, enhanced the secretion of potassium (Kessler, Hierholzer, Gurd & Pitts, 1959) decreased $T_m$ PAH and was without effect on total renal blood flow (Fig. 1).

Secondly, there was remarkable similarity between the action of chlorothiazide on the excretion of salt and water in the patient with diabetes insipidus (Crawford & Kennedy, 1959; de los Reyes *et al.* 1960) and in the isolated cat kidney perfused with blood collected from headless donors (Figs. 2, 3). The only difference in the effect of chlorothiazide on the kidneys of normal man and of the patient with diabetes insipidus was the

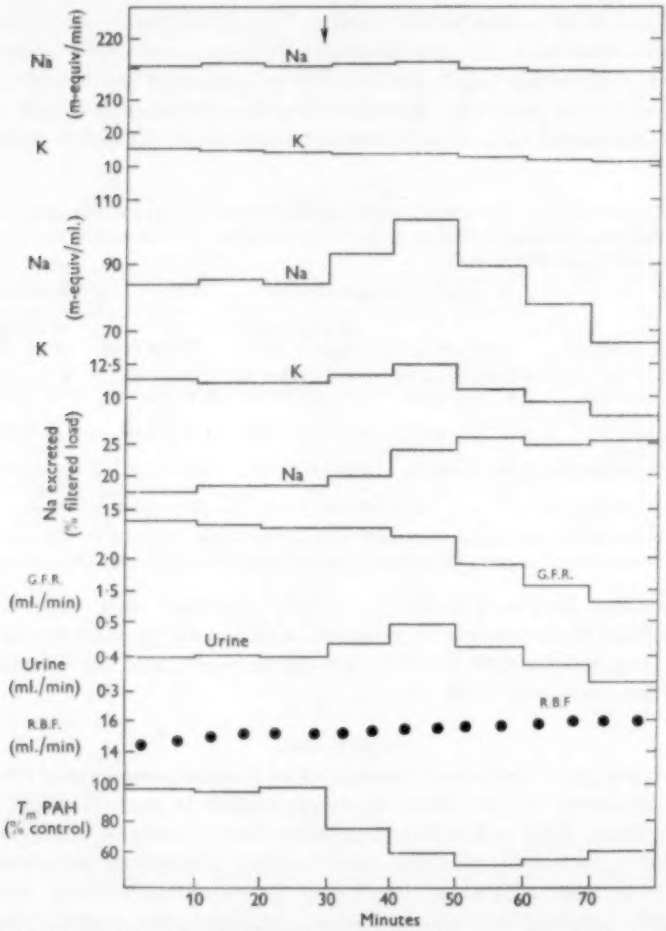



Fig. 2. The effect of chlorothiazide on the excretion of $Na^+$, $K^+$ and water by a cat's kidney, 9·2 g, perfused at 148 mm Hg with blood taken from a headless donor under chloralose anaesthesia. Ordinates and abscissae as in Fig. 1. Chlorothiazide, 10 mg/140 ml. blood given at arrow.

reduction in filtration rate that accompanied the effect of the drug in the diabetic (de los Reyes *et al.* 1960). Reduction in filtration rate was also a constant finding when kidneys perfused with blood from headless donors were treated with chlorothiazide (Figs. 2, 3); again it accounted for all the differences in the action of the drug on sodium, potassium and water excretion by isolated kidneys when perfused, on the one hand, with blood from intact donors (Fig. 1), and on the other, with blood from headless donors (Figs. 2, 3). The effect of chlorothiazide on fitration rate in the diabetic patient and isolated kidney perfused with blood from headless

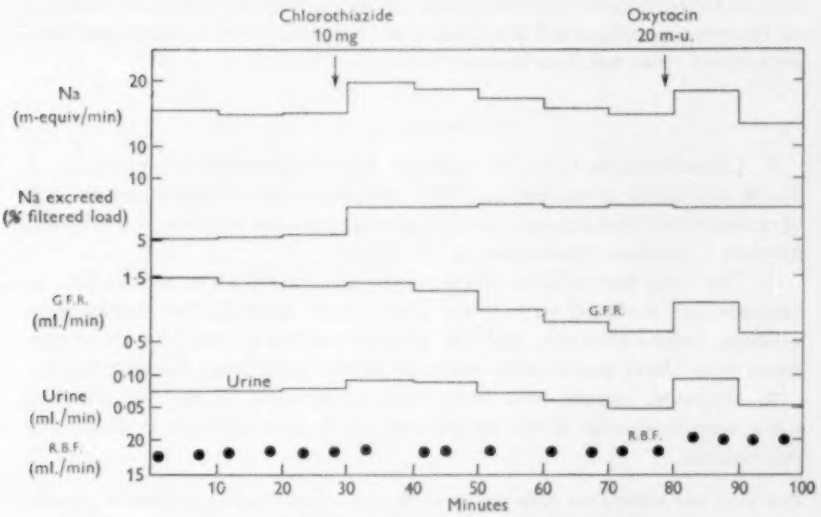



Fig. 3. Transient antagonism by oxytocin of the reduction in G.F.R. and urine flow caused by chlorothiazide in a cat kidney, 12·2 g, perfused at 146 mm Hg with blood taken from headless donors under chloralose anaesthesia. Ordinates, from above downward: Urinary excretion of Na; Na excreted (% filtered load); G.F.R.; urine volume; R.B.F. Abscissae, time. Chlorothiazide, 10 mg and oxytocin 20 m-u. added to 140 ml. perfusing blood at first and second arrows respectively.

donors cannot be attributed to lack of ADH for two reasons. First, Dávid *et al.* (1960) found that chlorothiazide failed to produce antidiuresis in psychic polydypsics, in whom the circulating concentration of ADH is very low; secondly, the cat kidney perfused under chloralose anaesthesia is remarkably insensitive to ADH (Davey & Lockett, 1960), yet the differences between the diabetic and non-diabetic response to chlorothiazide were apparent in these preparations. Neither can the reduction of filtration rate characterizing the action of chlorothiazide in diabetes

insipidus be attributed to lack of oxytocin, because 20 m-u./150 ml. blood produced only transient antagonism of this reduction (Fig. 3).

It is therefore concluded that the patient with diabetes insipidus and blood drawn from headless animals both lack some other substance or substances, stable during the night in blood at 7° C. and surviving 5 hr of perfusion through a heart-lung-kidney preparation without excretion or destruction, which conditions some part of the renal vascular bed to withstand the vascular action of chlorothiazide. This same substance or substances may be concerned with the maintenance of normal sodium reabsorption, since kidneys perfused with blood from headless donors leak sodium at more than the normal rate (Table 1). Neither lack of oxytocin or of vasopressin influenced this steady sodium leak from kidneys perfused with blood from headless donors (Table 1).

## SUMMARY

1. Chlorothiazide 10 mg/140–150 ml. blood, increased the excretion of Na, K and water, decreased $T_m$ PAH, and caused no change in total R.B.F. of isolated perfused cat kidneys. These changes were evident in 5 min and reached a sustained maximum in 20–30 min.

2. The drug was without effect on glomerular filtration rate (G.F.R.) in preparations perfused with blood from intact animals, but caused progressive decline in G.F.R. and the minute volume of urine in those perfused with blood drawn after exclusion of the head from the circulation.

3. Oxytocin caused only short-lived antagonism of this reduction in G.F.R. and no change in the proportion of the filtered Na load which was reabsorbed.

This work was undertaken while one of us (T. de L.) was receiving a grant for personal maintenance during training in research from Cipla Laboratories, Bombay.

## REFERENCES

Bayliss, R. I. S., Marrack, D., Perkis, J., Rees, J. R. & Silva, J. F. (1958). The use of chlorothiazide in the treatment of edema: a comparison with other diuretic agents. *Ann. N.Y. Acad. Sci.* **71**, 442–449.

Beyer, K. H. (1958). The mechanism of action of chlorothiazide. *Ann. N.Y. Acad. Sci.* **71**, 363–379.

Crawford, J. D. & Kennedy, G. C. (1959). Chlorothiazide in diabetes insipidus. *Nature, Lond.*, **183**, 891–892.

Davey, M. J. & Lockett, M. F. (1960). Actions and interactions of aldosterone monacetate and neurohypophysial hormones on the isolated cat kidney. *J. Physiol.* **152**, 206–219.

Dávid, M. A., Lázlo, F. A. & Kovács, K. (1960). Contrasting effect of chlorothiazide in diabetes insipidus and psychic polydypsia. *Lancet*, **279**, 210–211.

de los Reyes, M. P., Gómez, R. & Bradford, I. (1960). Hydrochlorothiazide and diabetes insipidus. *Lancet*, **278**, 650.

Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*, 625–653. London: Churchill.

KENNEDY, G. C. & CRAWFORD, J. D. (1959). Treatment of diabetes insipidus with hydrochlorothiazide. *Lancet*, **276**, 866–867.

KESSLER, R. H., HIERHOLZER, K., GURD, R. S. & PITTS, R. F. (1959). Localisation of the action of chlorothiazide in the nephron of the dog. *Amer. J. Physiol* **196**, 1346–1351.

LARAGH, J. H. (1958). Some effects of chlorothiazide on electrolyte metabolism and its use in edematous states. *Ann. N.Y. Acad. Sci.* **71**, 409–419.

SMITH, H. W., FINKELSTEIN, N., ALIMINOSA, L., CRAWFORD, B. & GRABER, M. (1945). The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dogs and man. *J. clin. Invest.* **24**, 388–404.

*J. Physiol.* (1961), **157**, *pp.* 462–474
*With 5 text-figures*
*Printed in Great Britain*

# BRONCHIAL CIRCULATION AND PULMONARY VASOMOTOR NERVE RESPONSES IN ISOLATED PERFUSED LUNGS

BY P. R. ALLISON, I. DE BURGH DALY AND B. A. WAALER*

*From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge, and the University Laboratory of Physiology, Oxford*

(*Received* 23 *January* 1961)

It is well known that pulmonary vasomotor responses to nerve stimulations in isolated lungs of the dog perfused through the pulmonary circulation alone are ephemeral or absent (for literature see Daly, 1958). It was pointed out by Berry, Brailsford & Daly (1931) that failure to obtain clear evidence of pulmonary vasomotor nerve activity in such perfused preparations might be due to an insufficient blood supply to the nerves, which Reisseisen (1822) had shown were supplied by the bronchial arterial system. For this reason they advocated additional perfusion of the bronchial vascular system. This method of perfusion was used by Daly & von Euler (1932), who obtained well marked pulmonary vasomotor responses to nerve stimulations over a period of several hours. Since that time it has been repeatedly observed that such responses are only obtained during bronchial circulation perfusion and are extinguished if this perfusion is interrupted. These observations, however, provided little evidence of the underlying mechanism reponsible for the maintenance of pulmonary vasomotor nerve function by bronchial circulation perfusion. The investigation here described on the lungs of the dog relates to (1) the survival of pulmonary vasomotor nerves during bronchial circulation ischaemia, (2) the bronchial circulation perfusion time required to recover pulmonary vasomotor nerve function after varying periods of ischaemia, (3) the duration of bronchial circulation ischaemia which extinguishes pulmonary vasomotor nerve responses, (4) the effect of reducing the partial pressure of oxygen ($pO_2$) in the blood perfusate on the responses to pulmonary vasomotor nerve stimulation, and (5) the minimal bronchial arterial pressure required to maintain pulmonary vasomotor nerve responses.

## METHODS

Mongrel dogs varying in weight from 14 to 29 kg were exsanguinated under local anaesthesia through a cannula inserted into the femoral artery, after premedication with morphine hydrochloride (2 mg/kg subcutaneously). Atropine sulphate (1 mg) and heparin (Boots

* Research Fellow, Norwegian Research Council for Science and the Humanities.

powdered heparin 3 mg/kg dissolved in distilled water) were injected intravenously some minutes before exsanguination. The blood was collected in a glass cylinder containing an amount of heparin approximately equal to twice that injected intravenously, the total dose of heparin used being 9 mg/kg body weight. Penicillin (Crystapen, crystalline sodium Penicillin G, Glaxo, 500 i.u./100 ml. blood) was added to the blood, which was then filtered through one layer of linen (mesh 50 $\mu$ by 250 $\mu$) resting on a stainless-steel mesh (250 $\mu$ by 250 $\mu$). The blood was stored at 36° C until used for perfusing the lungs.

In some experiments the left lung was perfused through both the pulmonary and bronchial circulations by the method described by Daly (1956). In others, either the left apical and cardiac lobes or the left lower lobe were perfused by a similar method but with a reduced volume of the blood reservoir (250 ml.) (Daly & Waaler, 1960).

All glass pieces were cleaned and brushed in tap water, soaked in chromosulphuric acid for 24 hr, rinsed ten times in running tap water and twice with distilled water. The rubber connexions were soaked in running tap water for several hours, boiled in 2% $NaHCO_3$, rinsed in running tap water and finally in distilled water.

The pulmonary circulation was perfused at a constant-volume blood inflow or at a constant head of pressure at 14–22 mm Hg, and the bronchial circulation, including the vascular territory supplying the nerves to be stimulated, at a mean pressure of 80–140 mm Hg. The pulmonary arterial pressure was recorded with a Marey tambour and the left atrial blood outflow with a modified Gaddum blood-flow recorder. The left atrial pressure was recorded by a blood manometer, the top of which was connected to a piston recorder, or taken as the difference in levels between the mouth of the left atrial cannula and the blood as it spilt over into the flow recorder. This latter measurement was found by experiment to be a few millimetres of blood lower than the true left atrial pressure at the blood flow used.

The pulmonary vascular resistance (PVR) was calculated according to the formula

$$\text{PVR} = \frac{\text{Pulmonary arterial pressure (mm Hg)} - \text{Left atrial pressure (mm Hg)}}{\text{Left atrial flow (l./min)} \times x/\text{m}^2},$$

where $x$ equals the ratio of total lung weight to the weight of the perfused lobes, as given by Rahn & Ross (1957). When this formula is used, the PVR for normal resting dogs ranges from 1·7 to 7·5.

*Lung ventilation.* The lung lobes were ventilated by starting a Starling 'Ideal' pump (C. F. Palmer Ltd.) at a peak pressure of 80–100 mm $H_2O$ with pure $O_2$, 6% $CO_2$ in $O_2$ or 6% $CO_2$ in air. Hypoxaemia was produced in seven tests by changing the ventilation from 6% $CO_2$ in $O_2$ to 6% $CO_2$ in $N_2$, and in one test by changing the ventilation from $O_2$ to $N_2$. The tidal air was measured by the method of Konzett & Rössler (1940).

*Tests for pulmonary vasomotor activity.* The upper thoracic sympathetic chain, the stellate ganglion, the ventral branch of the ansa subclavia or the left thoracic vagosympathetic nerve was stimulated by square-wave impulses derived from an Attree (1950) stimulator. The predominant response to stimulation of the sympathetic fibres in this nerve pathway is pulmonary vasoconstriction (Daly, Duke, Hebb & Weatherall, 1948).

Atropine sulphate equivalent to about 0·05 mg/kg body weight was injected intravenously before exsanguination, or added to the blood reservoir during perfusion, in order to suppress any passive pulmonary circulatory effects due to 'bronchoconstrictor' responses during stimulation of the left thoracic vagosympathetic nerve.

*Blood analysis.* Blood samples were taken from the pulmonary artery inflow tubing during the course of each experiment. The temperature of the blood perfusate at the time of sampling, usually about 34–35° C, was registered. The samples were stored under paraffin oil at 4° C until the pH measurement and blood gas analysis could be carried out.

The oxygen capacity, the degree of oxygen saturation and the carbon dioxide content of the blood were determined with a modified Haldane blood gas apparatus (Shaw, 1959) by the method of Courtice & Douglas (1947).

Blood pH was measured at room temperature with a Pye 'Master' pH meter (Cat. No. 11068, W. G. Pye and Co.). The apparatus was standardized before each determination by a buffer of pH 7·01 at 20° C. Duplicated estimations agreed within 0·02 pH units or less. The values of blood pH resulting from these measurements at room temperature were converted to pH values corresponding to the blood temperatures in the preparations at the time of sampling by using the temperature coefficient of Rosenthal (1948). Some pH measurements were carried out at 35° C with a 'Vibron' 33B electrometer (Electronic Instruments Ltd.) in conjunction with a 'Vibron' C 33B pH measuring unit. The accuracy of these measurements was at least as great as those done with the 'Master' pH-meter.

Blood $pO_2$ values were calculated from the oxygen saturation values using the nomogram and the correction factors (for pH and temperature) given by Severinghaus (1958) on p. 73 in *Handbook of Respiration*, assuming that the oxyhaemoglobin dissociation curve for dog follows that of man. Maximal errors in our calculated $pO_2$ values, arising from errors in the measurements needed (oxygen saturation, blood pH and blood temperature), could be calculated to be of the order of $\pm 1$ mm Hg and $\pm 3$ mm Hg in the $pO_2$ regions of 25 and 50 mm Hg respectively.

$pCO_2$ values were calculated from the values for $CO_2$ content, pH, oxygen capacity and percentage oxygen saturation, by the formula:

$$pCO_2 = \frac{CO_2}{S(10^{(pH-pK')}+1)}$$

(Severinghaus, Stupfel & Bradley, 1956*a*). The plasma $CO_2$ content (mM/l.) was calculated by multiplying the value for whole blood by the factor '*f*' read from the nomogram of Van Slyke & Sendroy (1928). The solubility factor, *S*, was read from the table of Severinghaus *et al.* (1956*a*), and the value for pK′ from the nomogram of Severinghaus, Stupfel & Bradley (1956*b*).

## RESULTS

All the procedures necessary for the separation from the thorax of the reduced systemic circulation, together with the heart and lung lobes to be perfused, were carried out after exsanguination. The period of time which elapsed between exsanguination and the moment when pulmonary circulation perfusion, ventilation and bronchial circulation perfusion (in that order) were established, varied from 45 to 314 min (mean: 132 min) in 52 experiments.

*Survival of pulmonary vasomotor nerves during bronchial circulation ischaemia.* In most experiments perfusion of the pulmonary circulation and lung ventilation were started before perfusion of the bronchial circulation. Stimulation of the sympathetic nerve path or of the associated ganglia failed to produce a pulmonary vasopressor response before perfusion of the bronchial circulation. Within 2–3 min of the start of bronchial circulation perfusion small but definite pulmonary vasopressor responses to nerve stimulation were obtained. For any given strength of stimulus these reached a maximum after 22–35 min of perfusion. A more accurate assessment of the duration of bronchial circulation perfusion required to establish maximal responses to nerve stimulations could not be obtained because the stimuli were applied at intervals of 10 min. This interval of time was selected because it had been found by experience that it ensured

consistent pulmonary vasomotor responses to successive stimuli over a period of some hours. The records of Fig. 1, which are typical of a large number of experiments, show the absence of a pulmonary vasopressor nerve response before bronchial circulation perfusion (12.01 p.m.), a small response 2 min after the start of bronchial circulation perfusion (12.11 p.m.) and a maximal response 22 min after the start of perfusion (12.31 p.m.).

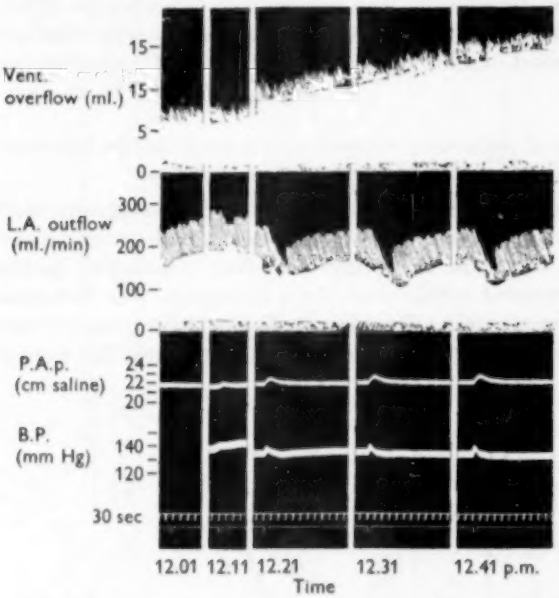



Fig. 1. Effect of stimulating sympathetic post-ganglionic nerve fibres (in left thoracic vagosympathetic nerve) on an atropinized perfused lung lobar preparation before and during additional perfusion of bronchial circulation. Dog: *f*, 17·0 kg, 0·75 m² body surface; perfusion of left apical and cardiac lobes at a constant head of pressure. Bronchial circulation perfusion started at 12.09 p.m. Stimulations during 15 sec periods, 10 V, square-wave impulses of 10 m-sec duration and frequency 47/sec.

Calculated total pulmonary vascular resistance (PVR) values before stimulations and at peak of vasoconstrictor responses: 12.01 p.m., 6·3–6·3; 12.11 p.m., 5·6–6·4; 12.21 p.m., 6·5–10·6; 12.31 p.m., 6·8–12·1; 12.41 p.m., 6·7–11·7. Vent. = ventilation. L.A. = left atrial. P.A.p. = pulmonary arterial pressure. B.P. = perfusion pressure of aorta and bronchial arteries.

These pulmonary vasopressor responses once established were obtainable during short periods of interruption of bronchial circulation perfusion (see Figs. 2 and 3), thus demonstrating that they were not caused by passive bronchial vascular effects on the pulmonary circulation. Since the vaso-

pressor responses occurred in the absence of changes in lung hindrance they were due to pulmonary vasoconstriction.

The experiment in Fig. 1 was carried out at constant head of perfusion pressure, and similar results were obtained in other preparations perfused at constant blood volume inflow.

In spite of the prolonged period of initial bronchial circulatory ischaemia, which in one experiment was deliberately extended to 314 min, well marked pulmonary vasoconstrictor responses to nerve stimulation were always obtained within 35 min of the start of bronchial circulation perfusion.

*Extinction of pulmonary vasomotor nerve responses by interruption of bronchial circulation perfusion*

Interruption of bronchial circulation perfusion caused a gradual decrease and, finally, extinction of the responses to sympathetic nerve stimulation within 9–38 min. On restarting bronchial circulation perfusion the responses recovered within from 7 to 40–50 min. The reduction in the response occurred within a few minutes of bronchial circulation interruption. This is shown in Fig. 2 in two experiments in which the responses were

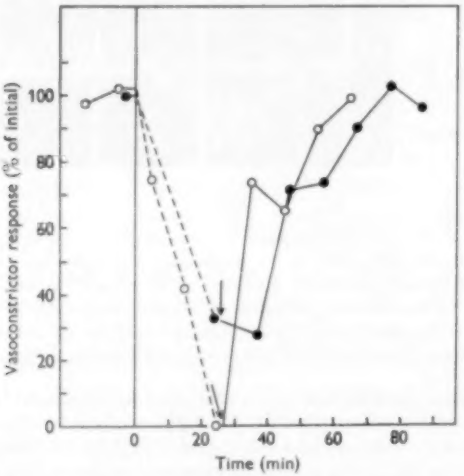



Fig. 2. Effect of interruption of bronchial-circulation perfusion on pulmonary vasoconstrictor responses to stimulation of post-ganglionic sympathetic nerve fibres (in left thoracic vagosympathetic nerve) in two isolated perfused left lung lobar preparations of the dog. Responses expressed as % of initial values. Each point represents the response to one stimulation. Bronchial circulation perfusion interrupted at zero time, restarted at the arrows; the broken lines thus indicating periods of no bronchial circulation perfusion.

due to stimulation of the post-ganglionic fibres in the left thoracic vago-sympathetic nerve. In one experiment (open circles) the stimuli were applied at 10-min intervals and extinction of the response occurred 24 min after interruption of bronchial circulation perfusion; full recovery of the response was obtained 38 min after restarting perfusion. In the other experiment shown stimuli were withheld until bronchial circulation

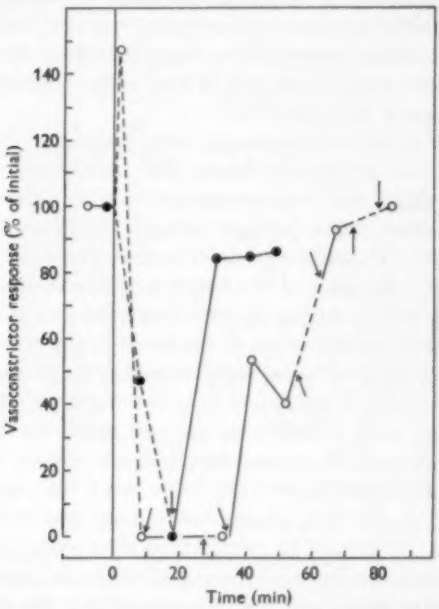



Fig. 3. Effect of interruption of bronchial circulation perfusion on pulmonary vasoconstrictor responses to stimulation of preganglionic sympathetic nerve fibres or of a sympathetic ganglion. Isolated perfused left lung preparations of the dog. ○ an experiment in which the left sympathetic chain was stimulated; ● an experiment in which the left stellate ganglion was stimulated. Responses expressed as % of the initial values. Each point represents the response to one stimulation. Bronchial circulation perfusion interrupted at zero time and at all arrows pointing upwards, perfusion restarted again at all arrows pointing downwards, the broken lines thus indicating periods of no bronchial circulation perfusion.

ischaemia had been established for 23 min, after which the response obtained was 33 % of its initial value. Restarting perfusion of the bronchial circulation produced, after a preliminary fall, full recovery of the response within 40–50 min. Similar results were obtained in three additional experiments of the same type.

The extinction of the responses during bronchial circulation interruption

to stimulation of the upper thoracic sympathetic chain and of the stellate ganglion was tested in three experiments of which two are shown in Fig. 3. It will be seen that the responses were abolished in 9 and 18 min, respectively, rather earlier than the extinction of the responses to stimulation of the post-ganglionic fibres in the thoracic vagosympathetic nerve (Fig. 2). Another feature of these experiments was the appearance of a supernormal response to the first stimulus following bronchial circulation interruption (Fig. 3). Such an initial supernormal response was observed in two out of five bronchial circulation interruption tests in which the sympathetic chain was stimulated and in one out of four tests in which the thoracic vagosympathetic nerve was stimulated.

*Hypoxaemia and pulmonary vasomotor nerve responses.* In the perfusion system used the two pumps perfusing the pulmonary and bronchial circulations—including the extrapulmonary nerves—drew their blood from a single reservoir. After passage through the lungs the blood from the left atrium flowed through the blood outflow recorder to be collected in the reservoir, and this part of the blood circuit was open to the atmosphere. It was found that during $N_2$ ventilation the $pO_2$ of the blood from the left atrium was lower than that of the blood in the reservoir. This was because the blood picked up some oxygen during its passage through the flow recorder and whilst it spilt over into the reservoir. For this reason the blood perfusing both circulations did not reach the low $pO_2$ values which might be expected if a closed blood-circuit system had been used. Thus, in eight tests in which the lung lobes were ventilated with $N_2$ for periods of 23–76 min the $pO_2$ of the blood only reached 26–38 mm Hg (mean 32 mm Hg). It should be emphasized that under these conditions of perfusion $N_2$ ventilation reduced the $pO_2$ of the blood circulating through all the tissues of the lungs and also that supplying the extrapulmonary nerves. In seven out of the eight tests mentioned above $N_2$ ventilation reduced the pulmonary vasoconstrictor responses to stimulation of the sympathetic post-ganglionic fibres in the left thoracic vagosympathetic nerve. In the remaining experiment the responses were not altered. Figure 4 illustrates the results of two of these tests. In one, $N_2$ ventilation for 30 min produced a moderate reduction in the vasoconstrictor response to nerve stimulation and complete recovery followed ventilation with 6% $CO_2$ in $O_2$. In the other the reduction in the response was much greater and recovery was incomplete.

There was no correlation between the reduction in pulmonary vascular responses to nerve stimulation during $N_2$ ventilation and the relatively small changes in pH and blood $pCO_2$ which accompanied the fall in blood $pO_2$.

The two tests in Fig. 4 are given as examples because $N_2$ ventilation

itself produced no appreciable changes in PVR. In other experiments, however, this type of preparation responded to $N_2$ ventilation by a fall in PVR. Such a *fall* occurred in five out of the eight tests described above, and in these five experiments also the vasomotor responses to nerve stimulation were reduced, the percentage reduction falling between those shown in Fig. 4. It is realized that the pulmonary vascular depressor effect of hypoxia, which occurred in these experiments, is contrary to the

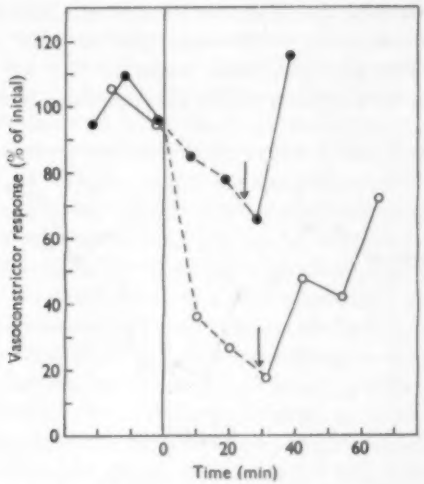



Fig. 4. Effect of temporary $N_2$ ventilation on pulmonary vasoconstrictor responses to sympathetic nerve fibre stimulation in two isolated perfused left lung lobar preparations of the dog. Post-ganglionic fibres of left thoracic vagosympathetic nerve stimulated. Responses expressed as % of the initial values. Each point represents the response to one stimulation. At zero time ventilation changed from 6 % $CO_2$ in $O_2$ to 6 % $CO_2$ in $N_2$. Ventilation changed back to 6 % $CO_2$ in $O_2$ at arrows, the broken lines thus representing periods of $N_2$ ventilation.

findings of the majority of other investigators. This is all the more surprising since a vasopressor response to $N_2$ ventilation was never observed in our experiments. Our perfusion technique differed from that of others who have investigated the effect of hypoxia on the circulation in isolated lung preparations, in that the initial period of pulmonary circulation ischaemia was relatively long and that additional perfusion of the bronchial circulation was carried out. To what extent these factors have determined the vasomotor response to $N_2$ ventilation is being explored, and will not be further discussed here since it is not obviously relevant to our present examination of the effect of $N_2$ ventilation on pulmonary vasomotor nerve

responses. These responses were reduced during $N_2$ ventilation irrespective of the direct effect of $N_2$ ventilation on the pulmonary circulation.

In one experiment the addition of KCN to the perfusate in the reservoir, to give a blood concentration of 0·1 mg/ml., abolished the pulmonary vasomotor nerve responses within 25 min.

*Systemic arterial pressure and pulmonary vasomotor nerve responses*

Three experiments were performed to determine the minimal arterial pressure in the bronchial circulation and in the vessels supplying the extra-pulmonary nerves required to maintain normal pulmonary vasomotor nerve response. The post-ganglionic fibres in the left thoracic vago-sympathetic nerve were stimulated for this purpose. It was found during

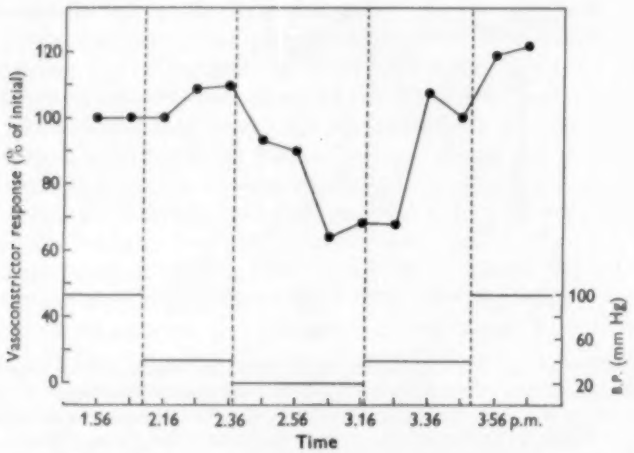



Fig. 5. Effect of altering the perfusion pressure (B.P.) in the bronchial arteries (and aorta) on pulmonary vasoconstrictor responses to sympathetic nerve fibre stimulation (post-ganglionic fibres of left thoracic vagosympathetic nerve) in an isolated perfused left lung lobar preparation of the dog. The points in the upper tracing represent responses to stimulations at 10 min intervals. Responses expressed as % of initial values. The lower lines indicate bronchial circulation perfusion pressure.

two separate tests that a perfusion pressure of 40 mm Hg sufficed to maintain normal responses, but in each test reducing the pressure below this value led to a reduction in the pulmonary vasomotor nerve responses. One of these experiments is illustrated in Fig. 5. A third test on lowered bronchial circulation perfusion pressure showed reduced vasomotor nerve responses when the pressure was brought down to 70 mm Hg or less.

## DISCUSSION

The results confirm that in isolated perfused lungs of the dog perfusion of the bronchial circulation is essential for the maintenance of pulmonary vasoconstrictor responses to sympathetic nerve stimulation. The observations that an aortic (bronchial arterial) pressure as low as 30–40 mm Hg or intermittent interruption of bronchial circulation perfusion for 10 min alternating with 10-min periods of perfusion (Fig. 3) can suffice to maintain the responses to their full value indicates that only a relatively small blood flow in the bronchial circulation is necessary to maintain functional activity of the nerves. This is in agreement with the findings of Eccles (1935) and Bargeton (1938) in studies on the superior cervical ganglion.

It seems reasonable to conclude from the experiments with lowered oxygen tension that one of the functions of the bronchial circulation is the maintenance of an oxygen supply to the pulmonary nerves and/or the neuromuscular apparatus. We have little evidence as to the possible role of other blood components supplied by, or removed by, the bronchial circulation. It was, however, observed that addition to the blood reservoir of 'new' blood (i.e. blood which has been stored in a thermostat at 36° C since the death of the animal) sometimes caused a full or a partial restoration of vasomotor responses to nerve stimulations in preparations in which such responses had started to decline. The oxygenation of the perfusate was adequate throughout these experiments, so that other blood components than oxygen must have been responsible for the effect. We have at present no indication as to the nature of this potentiating effect of 'new' blood.

In two experiments it was observed that a stimulus applied to the sympathetic chain or the stellate ganglion just after the bronchial circulation perfusion had been interrupted caused a larger pulmonary vasoconstrictor response than the control value (Fig. 3). The number of experiments is too small to assess the significance of this observation, which, nevertheless, recalls that Bronk & Larrabee (1937) observed an increase of the electrical response and a persistent facilitation in a ganglion during the initial period after it was deprived of its blood supply.

It should be emphasized that the method of perfusion used differed in two respects from that generally practised in setting up isolated perfused lungs. First, there was a prolonged period of pulmonary circulation ischaemia before the start of its perfusion, and secondly all the tests were made during bronchial circulation perfusion, or after a short interruption of such perfusion. How far the initial period of ischaemia affected the pulmonary vascular responses to sympathetic nerve stimulation is uncertain, because under the conditions of the experiment the pulmonary

vascular response before the period of circulatory ischaemia could not be assessed. The experiments show, however, that some of the sympathetic vasoconstrictor fibres are strongly resistant to ischaemia of the bronchial vascular system, in that after prolonged periods of such ischaemia their functional activity can be gradually restored by starting up bronchial circulation perfusion. That the revived nerve fibres in most of the experiments probably represent a high proportion of all those present in the nerve trunk stimulated is suggested by the responses being no smaller than those obtained by Daly *et al.* (1948) in the perfused living animal preparation, in which bronchial circulation ischaemia was absent and there was only a short period of pulmonary circulation ischaemia whilst the preparation was being made.

A puzzling feature of the present series of experiments has been the absence of sympathetic vasodilator responses to nerve stimulations. Such responses, although relatively rare, can be evoked in the perfused living animal (Daly *et al.* 1948; Daly & Daly, 1959). The explanation may be that these vasodilator fibres are less resistant to bronchial circulation ischaemia than the sympathetic pulmonary vasoconstrictor fibres. Since all the perfused preparations were atropinized it was not feasible to examine the effects of ischaemia or of ventilation hypoxia on the atropine-sensitive pulmonary vasodilator fibres described by Daly & Hebb (1952).

The fact that the pulmonary vascular resistance was in the majority of experiments within normal limits for periods of perfusion of 4 hr, during which consistent pulmonary vasoconstrictor nerve responses were obtained, indicates a stability of the pulmonary vascular bed the greater part of which was being perfused. These consistent responses were obtained in experiments in which the lung hindrance gradually increased, thus suggesting that interstitial oedema was not an important factor in determining the responses.

The relation between the rate of decay of pulmonary vasoconstrictor responses and the duration of interruption of the bronchial circulation is of particular interest, since in testing for pulmonary vasomotor nerve activity it is essential to carry out some of the nerve stimulations during temporary interruption of bronchial circulation. Persistence of the pulmonary vasomotor responses during such interruptions excludes the possibility that they are due to passive effects on the pulmonary vascular bed caused by vasomotor changes in the bronchial vascular system. Temporary bronchial circulation interruptions usually lasted for 1–3 min only, and the observations reported indicate that during such periods the vasoconstrictor response to stimulation of post-ganglionic sympathetic fibres can be expected to be reduced by 15% or less.

SUMMARY

1. In atropinized perfused left lung lobe preparations of the dog, with additional perfusion of the bronchial circulation and of the vascular territories supplying the thoracic pulmonary nerves, stimulation of the nerve pathway carrying pulmonary sympathetic fibres (sympathetic chain, stellate or middle cervical ganglion and thoracic vagosympathetic nerve) produced vasoconstrictor responses.

2. The constrictor response was extinguished in 10–35 min by interruption of bronchial circulation perfusion and recovered in about the same period on restarting this perfusion.

3. Ventilation with 6% $CO_2$ in $N_2$, which reduced the $pO_2$ of the perfusate to 26–38 mm Hg, diminished but did not extinguish the vasoconstrictor response to sympathetic nerve stimulation. This diminution in response could not be correlated with the small changes in $pCO_2$ and pH which accompanied the changes in $pO_2$.

4. Bronchial arterial pressures below 40 mm Hg caused a diminution in the pulmonary vasoconstrictor response to sympathetic nerve stimulation in two out of three experiments.

5. Some of the pulmonary vasoconstrictor nerve fibres survive periods of bronchial circulation ischaemia lasting 5 hr.

Our thanks are due to Professor E. G. T. Liddell, F.R.S., and to Professor Sir Lindor Brown, F.R.S., for their kindness in granting us laboratory facilities, and to Mr S. B. Cross, Mrs Jennifer Barrett, Miss Jean Clough, Mr Nolan Howland and Miss Diana Jones for technical assistance. This work was supported by the Agricultural Research Council and by a grant from the Wellcome Trust to one of us (I.deB.D). B.A.W. was in receipt of personal grants from the Norwegian Research Council for Science and the Humanities, the University of Oslo, and Dr A. Malthes Legat.

REFERENCES

ATTREE, V. H. (1950). An electronic stimulator for biological research. *J. Sci. Instrum.* **27**, 43–47.

BARGETON, D. (1938). Some effects of acute anemia on the transmission of impulses through a sympathetic ganglion. *Amer. J. Physiol.* **121**, 261–269.

BERRY, J. L., BRAILSFORD, J. F. & DALY, I. DE B. (1931). The bronchial vascular system in the dog. *Proc. Roy. Soc.* B, **109**, 214–228.

BRONK, D. W. & LARRABEE, M. G. (1937). The effects of activity and altered circulation on ganglionic transmission. *Amer. J. Physiol.* **119**, 279.

COURTICE, F. C. & DOUGLAS, C. G. (1947). The ferricyanide method of blood-gas analysis. *J. Physiol.* **105**, 345–356.

DALY, I. DE B. (1956). Pulmonary vascular responses in an innervated isolated perfused left lung preparation. *J. Physiol.* **132**, 42–43*P*.

DALY, I. DE B. (1958). Intrinsic mechanisms of the lung. *Quart. J. exp. Physiol.* **43**, 2–26.

DALY, I. DE B. & DALY, M. DE B. (1959). The effects of stimulation of the carotid sinus baroreceptors on the pulmonary vascular bed in the dog. *J. Physiol.* **148**, 220–226.

DALY, I. DE B., DUKE, H., HEBB, C. O. & WEATHERALL, J. (1948). Pulmonary vasomotor fibres in the sympathetic chain and its associated ganglia in the dog. *Quart. J. exp. Physiol.* **34**, 285–313.

DALY, I. DE B. & HEBB, C. (1952). Pulmonary vasomotor fibres in the cervical vagosympathetic nerve of the dog. *Quart. J. exp. Physiol.* **37**, 19–43.

DALY, I. DE B. & VON EULER, U. S. (1932). The functional activity of the vasomotor nerves to the lungs in the dog. *Proc. Roy. Soc.* B, **110**, 92–111.

DALY, I. DE B. & WAALER, B. A. (1960). Bronchial circulation and pulmonary vascular resistance (PVR) in isolated perfused lung lobes. *J. Physiol.* **153**, 14*P*.

ECCLES, J. C. (1935). The action potential of the superior cervical ganglion. *J. Physiol.* **85**, 179–206.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. exp. Path. Pharmak.* **195**, 71–74.

RAHN, H. & ROSS, B. B. (1957). Bronchial tree casts, lobe weights and anatomical dead space measurements in the dog's lung. *J. appl. Physiol.* **10**, 154–157.

REISSEISEN, F. D. (1822). *Über den Bau der Lungen*, Berlin: Rücker.

ROSENTHAL, T. B. (1948). The effect of temperature on the pH of blood and plasma *in vitro*. *J. biol. Chem.* **173**, 25–30.

SEVERINGHAUS, J. W. (1958) in *Handbook of Respiration*, 1st ed. p. 73. Philadelphia: W. B. Saunders Company.

SEVERINGHAUS, J. W., STUPFEL, M. & BRADLEY, A. F. (1956*a*). Accuracy of blood pH and $P_{CO_2}$ determinations. *J. appl. Physiol.* **9**, 189–196.

SEVERINGHAUS, J. W., STUPFEL, M. & BRADLEY, A. F. (1956*b*). Variations of serum carbonic acid pK′ with pH and temperature. *J. appl. Physiol.* **9**, 197–200.

SHAW, D. G. (1959). *Some factors affecting respiration in man.* B.Sc. Thesis; University of Oxford.

VAN SLYKE, D. D. & SENDROY, J., JR., (1928). Studies of gas and electrolyte equilibria in blood. XV. Line charts for graphic calculations by the Henderson–Hasselbalch equation, and for calculating plasma carbon dioxide content from whole blood content. *J. biol. Chem.* **79**, 781–798.

*J. Physiol.* (1961), **157**, *pp.* 475–483
*With 4 text-figures*
*Printed in Great Britain*



# THE EFFECT OF BRADYKININ IN AN ISOLATED PERFUSED DOG LUNG PREPARATION

By B. A. WAALER*

*From the University Laboratory of Physiology, Oxford*

(*Received* 23 *January* 1961)

The polypeptide bradykinin has attracted much attention since the first description of its actions and properties by Rocha e Silva, Beraldo & Rosenfeld in 1949. Following the recent isolation of bradykinin (Elliott, Lewis & Horton, 1960*a*) and the determination of its structure (Elliott, Lewis & Horton, 1960*b*), it has been shown that the pure polypeptide possesses all the biological properties demonstrated for crude preparations (Elliott, Horton & Lewis, 1960).

In the systemic circulation bradykinin causes pronounced vasodilatation and increased capillary permeability in several species. However, the effect of bradykinin on the pulmonary vascular bed has not been examined. The only reported effect of bradykinin in the lungs is that of Collier, Holgate, Schachter & Shorley (1959), who showed that bradykinin causes bronchoconstriction when injected intravenously into guinea-pigs. Bronchoconstriction was also observed in isolated perfused guinea-pig lungs, although here much larger doses of bradykinin were necessary.

In the present experiments on an isolated perfused dog lung preparation, bradykinin caused vasodilatation of the pulmonary vascular bed, which, however, seemed to be less sensitive than other vascular fields. The airways of the isolated perfused dog lung preparation were never constricted and in some experiments were slightly dilated by bradykinin.

A preliminary communication on the findings has already been published (Waaler, 1960).

## METHODS

Mongrel dogs (17–25 kg) were premedicated with morphine (2 mg/kg) and injected intravenously with heparin (Boots powdered heparin, 3 mg/kg). In some of the experiments atropine sulphate (1 mg) was also injected intravenously. After inserting a cannula into a femoral artery under local anaesthesia exsanguination was carried out. During collection of the blood more heparin (6 mg/100 ml. blood) was added to the blood, which was then filtered through one layer of linen (mesh 50 $\mu$ by 250 $\mu$) and stored at 36° C until used undiluted for the subsequent perfusion.

*Perfusion.* Either the left apical together with the left cardiac lobe, or the left diaphragmatic lobe alone were used, perfusion of both the pulmonary and the bronchial

* Research Fellow, the Norwegian Research Council for Science and the Humanities.

circulation being carried out as previously described (Daly, 1956; Daly & Waaler, 1960). The pulmonary circulation was perfused either at constant volume inflow or at a constant head of perfusion pressure at 14–22 mm Hg. The reduced systemic circulation, including the bronchial vascular system, was perfused at constant volume inflow. This perfusion was carried out through a cannula inserted into the lower end of the thoracic part of aorta, the ascending aorta being clamped close to the heart. The main bronchial artery or arteries, which in the dog come off from the 5th–8th intercostal arteries on the right side, were segregated and preserved. The other arterial branches from the aorta were ligated. Some of the blood passing through the aorta, however, was short-circuited back to the blood reservoir through small arterial leaks or by means of a cannula inserted into an aortic branch. This arrangement prevented stagnation of blood in the aorta.

Cleaning of equipment and measurements of pressures and flow were carried out as described by Allison, Daly & Waaler (1961).

*Pulmonary vascular resistance.* The pulmonary vascular resistance (PVR) was calculated according to the formula:

$$\text{PVR} = \frac{\text{Pulmonary arterial pressure (mm Hg)} - \text{Left atrial pressure (mm Hg)}}{\text{Left atrial flow (l./min)} \times x/\text{m}^2 \text{ body surface}},$$

where $x$ equals the ratio of total lung weight to the weight of the perfused lobes, as given by Rahn & Ross (1957).

*Ventilation.* The lung lobes were ventilated with 6 % $CO_2$ in air by a Starling 'Ideal' pump at a peak inspiratory pressure of 80–90 mm of water. The ventilation overflow was measured by the method of Konzett & Rössler (1940).

*Bradykinin.* G. P. Lewis kindly supplied pure bradykinin (Elliott *et al.* 1960*a*) and also a partly purified sample ($P_3$). Both preparations were used diluted in NaCl solution 0·9 g/100 ml. The dilutions were made up freshly before each experiment. The bradykinin-containing solutions were injected into the pulmonary arterial inflow tubing or into the aortic inflow tubing, from which the bronchial arteries were being perfused. The injections were carried out with fine needles, the volumes (0·1–1·0 ml.) being kept constant for each experiment.

*Saliva* was collected from human subjects by their spitting into a container, and from the dogs by collecting the spontaneous flow after morphia medication. Both types of saliva were centrifuged at 1200 *g* for 30 min, the clear supernatant liquid being used.

## RESULTS

*Effect on pulmonary circulation.* The effect of injections of bradykinin into the pulmonary arterial inflow tubing was examined in eight atropinized and three non-atropinized preparations. The usual response to such an injection was a fall in PVR, which was obtained in nine experiments; no response was obtained with one preparation and an increase in PVR in another. The minimal effective dose producing a vasodilator response, as tested in three preparations, varied from 0·5 to 5$\mu$g of pure bradykinin. From 2 to 10$\mu$g produced the maximal dilator effect, giving a fall in PVR of from $\frac{1}{2}$ to 20 %. The dilator effect was evident in 15–30 sec, reached its maximum in 1–2 min and lasted for 2–20 min after the injection. When the injection of bradykinin was repeated several times, the responses diminished, even when the intervals between injections were as long as 15–20 min. This tachaphylaxis is illustrated in Fig. 1, which shows

the responses to three successive injections of pure bradykinin. With each subsequent injection the size and duration of the dilator response diminish. In the experiment of Fig. 1 the PVR was spontaneously and gradually falling during the period of the tests, as shown by the decline in perfusion

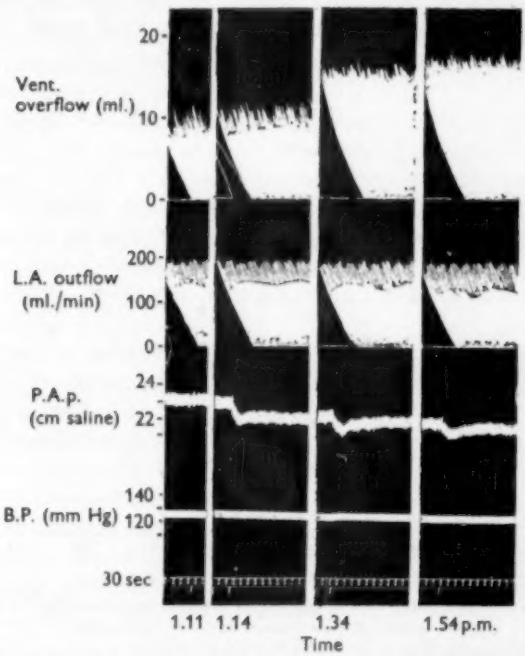



Fig. 1. Effect of successive injections of bradykinin into pulmonary artery of isolated perfused lung lobe preparation. Dog, *m*, 17 kg. Left apical and cardiac lobes perfused. Left atrial pressure = 6·5 cm saline. Injections into pulmonary arterial inflow tubing of 0·2 ml. 0·9% NaCl solution at 1.11 p.m., of 10 $\mu$g pure bradykinin (in 0·2 ml. 0·9% NaCl) at 1.14 p.m., 1.34 p.m. and 1.54 p.m. Vent = ventilation; L.A. = left atrial; P.A.p. = pulmonary arterial perfusion pressure; B.P. = perfusion pressure in reduced systemic circulation (bronchial circulation). Ventilation pump stroke = 77 ml. Pulmonary vascular resistance (PVR) range during these observations = 7·4–6·5.

pressure. A gradual fall in PVR during the initial 1–2 hr of perfusion is a usual feature of most experiments of this type.

Injections of the crude bradykinin $P_2$ caused vasomotor effects indistinguishable from those given by pure bradykinin, the relative potency of the crude material in this preparation being about 1/200 of that of the

pure polypeptide. This relationship agrees well with what Elliott *et al.* (1960) found when comparing pure bradykinin with $P_2$ on other biological preparations.

There was a tendency for bradykinin to cause more marked vasodilatation when the PVR was high than when it was low. This is illustrated in Fig. 2 which includes the maximal responses to bradykinin in ten experiments in which tests were carried out at different PVR levels. The high

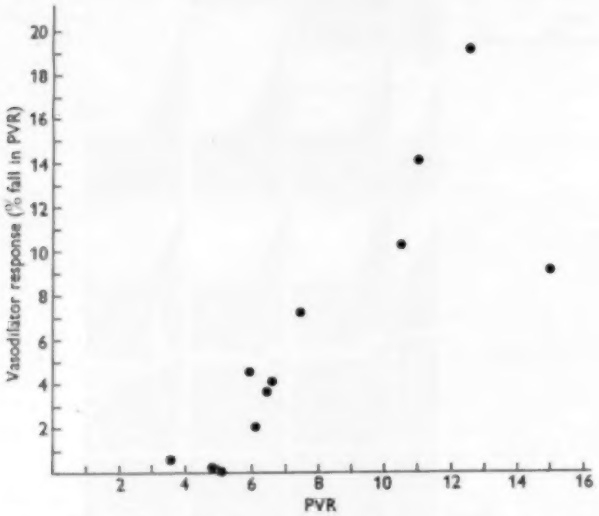



*Fig. 2.* Vasodilator responses to bradykinin in isolated perfused dog lung lobe preparations at various levels of pulmonary vascular resistance (PVR). Pure bradykinin or $P_2$ (partly purified substance) injected into pulmonary artery of preparations. The responses are the maximal ones obtained in 10 preparations, two of which are represented by two observations each.

PVR levels at which some of the tests were carried out occurred either during the pulmonary vasoconstriction which follows the start of bronchial circulation perfusion in a handled lung (Daly & Waaler, 1960) or at a late stage in an experiment, when the PVR tends to rise. Because of the tendency for tachaphylaxis, only one or two responses from each preparation are included in the figure. Where one preparation is represented by two observations, the last of them was separated from any previous injection of bradykinin by an interval of 1 hr or more. The vasomotor response to bradykinin was unaffected by atropine, the atropinized preparations giving vasodilator responses as great as those given by the non-atropinized ones.

*Effect on bronchial circulation.* Bradykinin caused a fall in the perfusion pressure of the reduced systemic circulation when injected into the aortic inflow tubing, or when added to the blood perfusate in the reservoir. Since the reduced circuit consisted of the aorta and the bronchial vascular bed only, this fall in pressure is interpreted as being due to a vasodilatation in the bronchial circulation. Because of the shunting of a considerable amount of blood from the aorta back to the reservoir (see Methods), this perfusion circuit did not allow more than a qualitative evaluation of its vasomotor responses.

*Effect on the airways.* Injections of pure bradykinin or of $P_2$ into the pulmonary artery either caused a small bronchodilatation (Fig. 3) or had no effect on the tidal air (Fig. 1). Small bronchodilator responses were seen in one out of three non-atropinized preparations and in four out of eight atropinized ones. The bronchodilator effect never caused more than a 5% increase in tidal air. Like the vascular effect of bradykinin the bronchodilator effect also diminished on repeated injections, as is illustrated in Fig. 3.

Injections of bradykinin through the aortic inflow tubing into the bronchial circulation also caused either a small bronchodilatation (one test) or no change in tidal air (two tests). Bradykinin did not cause bronchoconstriction in any of these experiments whether injected into the pulmonary or into the bronchial circulation.

*Effect of saliva on the isolated dog lung.* Dog saliva, known to contain a bradykinin-forming enzyme, caused a fall in PVR in five out of six tests when added to the blood perfusate. The response to the addition of 1–2 ml. of saliva was of the same magnitude as that obtained with injections of 10$\mu$g pure bradykinin into the pulmonary artery. This vasodilator effect occurred about 30 sec after the addition of saliva to the blood reservoir, and it lasted for 3–10 min. The preparation which responded to injections of pure bradykinin with pulmonary vasoconstriction gave a constrictor response also to the addition of dog saliva. In one of the tests there was a moderate bronchodilatation, in the remaining five no effect on the tidal air.

In contrast to dog saliva human saliva always caused a marked increase in PVR and a reduction in tidal air. The effects of dog and human saliva on the pulmonary and bronchial circulations and on the airways are shown in Fig. 4. Addition of 0·5–2 ml. of human saliva to the perfusion blood in nine other experiments gave results similar to those illustrated by the last observation in this figure. The vasoconstrictor response to human saliva started 0·5–1·25 min after the addition of the saliva to the blood reservoir, and reached its maximum 1·25–13 min after the addition. However, if the saliva was left in contact with cell-free plasma for 10–20 min, the

addition of this mixture to the reservoir caused a more rapidly developing vasoconstriction which reached its maximum within 1·5 min. In two of the ten tests with addition of human saliva the vasoconstrictor response was preceded by a short-lasting fall in PVR.

In preparations perfused at a constant head of pressure (as in Fig. 4) the flow did not recover after being reduced by the effect of added human saliva. In preparations perfused at constant volume inflow (pulsatile flow) the PVR usually returned to the previous value within 15–30 min.

When human saliva was added to the blood reservoir the bronchoconstrictor response started 0·5–4 min later, thus sometimes (as in the experiment of Fig. 4) being delayed in relation to the vasomotor response.

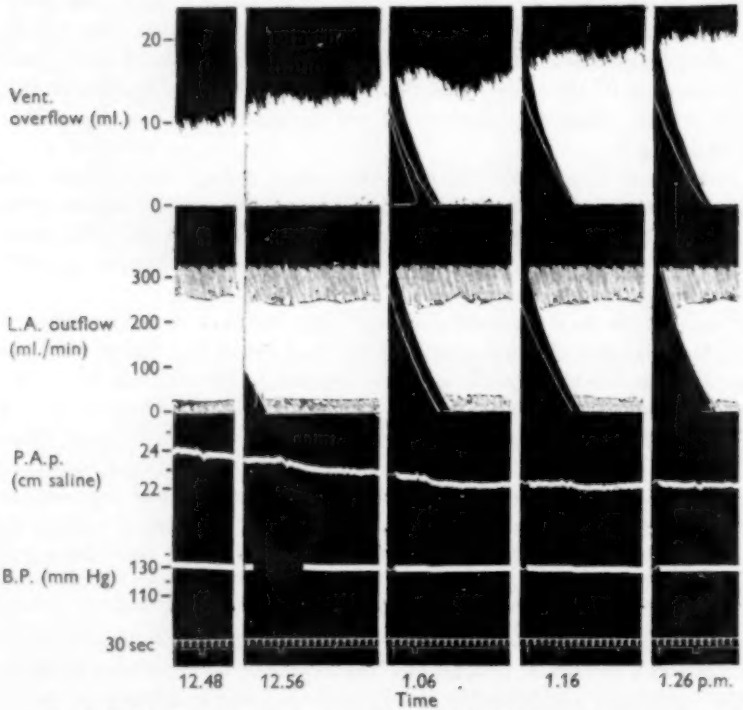



Fig. 3. Effect of successive injections of bradykinin ($P_2$) into pulmonary artery of isolated perfused lung lobe preparation. Dog, *m*, 23 kg. Left apical and cardiac lobes perfused. Left atrial pressure = 3·5 cm saline. Ventilation pump stroke = 85·5 ml. Injections into pulmonary arterial inflow tubing of 100 $\mu$g of $P_2$ at 12.48 p.m., 1 mg $P_2$ at 12.56 p.m., 5 mg $P_2$ at 1.06 p.m. and 1.16 p.m. and of 1 ml. 0·9% NaCl solution at 1·26 p.m. Each dose of $P_2$ dissolved in 1 ml. of 0·9% NaCl solution. Abbreviations as in Fig. 1. PVR range during these observations = 6·6–6·0.

Addition of human saliva to the blood perfusate usually had little effect on the bronchial circulation perfusion pressure (see Fig. 4), this pressure showing a small to moderate rise in three preparations, a fall in three and no change in another two. In the remaining two preparations tested the bronchial circulation was not perfused.

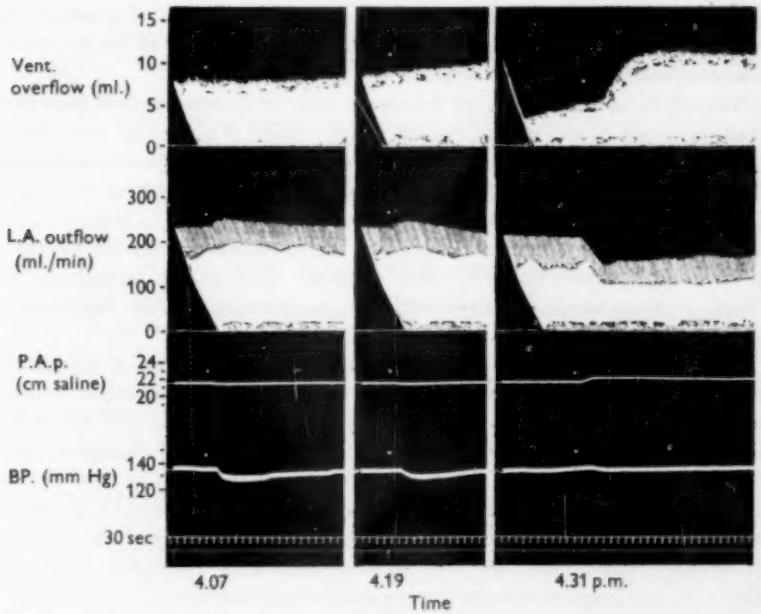



Fig. 4. Effect of addition of dog and human saliva to blood perfusate of isolated lung lobe preparation. Dog, *f*, 17 kg; 1 mg atropine sulphate intravenously before exsanguination. Left apical and cardiac lobes perfused. Left atrial pressure = 4 cm saline. Ventilation pump stroke = 44 ml. to 4.26 p.m., then reduced to 36 ml. Addition to blood reservoir of 1 ml. dog saliva at 4·07 p.m., 2 ml. dog saliva at 4.19 p.m. and 1 ml. human saliva at 4.31 p.m. Abbreviations as in Fig. 1. PVR range during these observations = 6·5–10·7.

## DISCUSSION

In isolated lung lobes of the dog, perfused with autologous blood through both the pulmonary and the bronchial circulations, vasodilatation was the predominant response of the pulmonary vascular bed to bradykinin injections. The reduction in pulmonary vascular resistance (PVR) caused by bradykinin was, however, never very marked. Bradykinin was also found to dilate the bronchial vascular system, but no comparison between this response and that of the pulmonary vascular bed was feasible, because

the systemic perfusion circuit was unsuitable for quantitative evaluations of vasomotor responses.

There are two explanations of the fact that the vasodilator effect of bradykinin in the pulmonary circulation was stronger at high levels of PVR than at low levels. One interpretation is that bradykinin may act more powerfully on vessels which have a greater vasoconstrictor tone than on more relaxed vessels, another that bradykinin may be acting selectively on types of vessels which remain open at the high PVR levels, whilst non-responsive vessels are closed down.

The effect of bradykinin on the airways in the isolated lung differs from that obtained in the guinea-pig, in which Collier *et al.* (1959) have found that bradykinin causes bronchoconstriction not only *in vivo* but with large doses in isolated perfused lungs as well. However, the recent finding of Collier & Shorley (1960), that a bronchodilator response to bradykinin in the guinea-pig may be unmasked when the bronchoconstrictor response is prevented by acetylsalicylic acid, suggests that in the guinea-pig too there is sometimes a bronchodilator component in the response to bradykinin.

The effect of dog saliva may be fully explained in terms of bradykinin formed when the bradykinin-forming enzyme in saliva comes into contact with plasma globulins. However, bradykinin formation cannot explain the completely different response to human saliva. Walaszek & Huggins (1959) have recently shown that when amylase, a constituent of human but not of dog saliva, is incubated with plasma proteins, a polypeptide is formed which has a vasoconstrictor effect. It is conceivable therefore, that the pulmonary vasoconstrictor and bronchoconstrictor responses to human saliva in our preparation are due to such a polypeptide.

## SUMMARY

1. Injections of pure bradykinin into the pulmonary artery of a perfused isolated dog lung preparation caused a small or moderate pulmonary vasodilatation. The vasodilatation was more marked at high than at low levels of pulmonary vascular resistance.

2. Whether the preparation was atropinized or not, bradykinin caused either a small bronchodilatation or had no effect on the tidal air.

3. Both the vasodilator and the bronchodilator responses to bradykinin were reduced on repeated injections.

4. Additions of dog saliva to the blood perfusate caused effects similar to those given by injections of large doses of pure bradykinin. Human saliva, however, caused marked pulmonary vasoconstriction and bronchoconstriction when added to the blood perfusate.

I wish to express my thanks to Professor I. de Burgh Daly, F.R.S. and to Dr G. P. Lewis for valuable advice and encouragement, to Professor E. G. T. Liddell, F.R.S. and to Professor Sir Lindor Brown, F.R.S. for their kindness in granting laboratory facilities, and to Mr Nolan Howland and Miss Diana Jones for technical assistance. This work was done during a period in which I received grants from the Norwegian Research Council for Science and the Humanities, from the University of Oslo, and from Dr A. Malthes Legacy.

## REFERENCES

Allison, P. R., Daly, I. de Burgh & Waaler, B. A. (1961). Bronchial circulation and pulmonary vasomotor nerve responses in isolated perfused lungs. *J. Physiol.* **157**, 462–474.

Collier, H. O. J., Holgate, J. A., Schachter, M. & Shorley, P. G. (1959). The bronchoconstrictor action of bradykinin in the guinea-pig. *Brit. J. Pharmacol.* **15**, 290–297.

Collier, H. O. J. & Shorley, P. G. (1960). Analgesic antipyretic drugs as antagonists of bradykinin. *Brit. J. Pharmacol.* **15**, 601–610.

Daly, I. de Burgh (1956). Pulmonary vascular responses in an innervated isolated perfused left lung preparation. *J. Physiol.* **132**, 42–43*P*.

Daly, I. de Burgh & Waaler, B. A. (1960). Bronchial circulation and pulmonary vascular resistance (PVR) in isolated perfused lung lobes. *J. Physiol.* **153**, 14*P*.

Elliott, D. F., Horton, E. W. & Lewis, G. P. (1960). Actions of pure bradykinin. *J. Physiol.* **153**, 473–480.

Elliott, D. F., Lewis, G. P. & Horton, E. W. (1960*a*). The isolation of bradykinin: A plasma kinin from ox blood. *Biochem. J.* **74**, 15–16*P*.

Elliott, D. F., Lewis, G. P. & Horton, E. W. (1960*b*). The structure of bradykinin. *Biochem. J.* **76**, 16*P*.

Konzett, H. & Rössler, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. exp. Path. Pharmak.* **195**, 71–74.

Rahn, H. & Ross, B. B. (1957). Bronchial tree casts, lobe weights and anatomical dead space measurements in the dog's lung. *J. appl. Physiol.* **10**, 154–157.

Rocha e Silva, M., Beraldo, W. T. & Rosenfeld, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Amer. J. Physiol.* **156**, 261–273.

Waaler, B. A. (1960). The effect of bradykinin in an isolated perfused dog lung preparation. *J. Physiol.* **154**, 57–58*P*.

Walaszek, E. J. & Huggins, C. G. (1959). Substance A: a hypertensive polypeptide from fraction IV-4 of plasma protein. *J. Pharmacol.* **126**, 258–263.

*J. Physiol.* (1961), **157**, *pp.* 484–503
*With 7 text-figures*
*Printed in Great Britain*

# ORIGIN AND BLOCKADE OF THE SYNAPTIC RESPONSES OF CURARIZED SYMPATHETIC GANGLIA

BY ROSAMOND M. ECCLES AND B. LIBET

*From the Departments of Physiology of the Australian National University, Canberra, Australia, and the University of California School of Medicine, San Francisco, U.S.A.*

(*Received* 23 *January* 1961)

In curarized sympathetic ganglia responding to preganglionic volleys, the initial negative (N) potential may be followed by a longer lasting positive (P) potential and, at least in the superior cervical ganglion of turtles and rabbits, by a prolonged late negative (LN) wave (Laporte & Lorente de Nó, 1950*b*; Eccles, 1952*b*). These slow P and LN waves are distinctly larger and more definite following a tetanic train of preganglionic volleys than after a single volley. They can occur in the absence of any ganglionic spike discharge and are affected not only by the curare-like substances and anti-cholinesterases (Eccles, 1952*a*, *b*) but also by certain other synaptic blocking agents to be reported below. (The symbols N, P, and LN will be used in this paper to refer only to potentials which do not require the discharge of a post-synaptic impulse for their production, as in Fig. 1, *II*, i.e. they will not include ganglion-cell after-potentials. The positivity following spike discharge in the uncurarized ganglion, as in Fig. 1, *I*, will not be referred to as a P-wave, but will be called after-positivity.) In this paper the question of the probable origins of these various components of the response seen in the curarized ganglion will be considered.

More specifically, the following points have been investigated in the present work. The distribution of the preganglionic fibres, among the lower and higher threshold fibre groups, in relation to the component of the response which is elicited, has been analysed. To test the possibility that a non-cholinergic pathway is involved in generating the P or LN responses, Botulinum toxin was applied to uncurarized ganglia. Ambache (1949, 1951), Burgen, Dickens & Zatman (1949), and Brooks (1956) have shown that this agent appears to paralyse specifically the release of acetylcholine without affecting responsiveness to applied acetylcholine (ACh) or adrenergic release, although the extent of this specificity has recently been questioned (Winbury, 1959). To help distinguish among

cholinergic actions, the effects of atropine were compared with those of curariform blockers. Numerous reports have indicated that adrenaline may be involved in transmission in sympathetic ganglia (see review by Goffart, 1957), and that there is a release of an adrenaline-like substance following preganglionic stimulation (Bülbring, 1944). Therefore the actions of adrenergic-blocking drugs and of certain related substances on the ganglionic potentials were investigated in the present work. It will be seen that the N, P, and LN components of the response of the curarized ganglion are best characterized as post-synaptic potentials which exhibit sometimes sharply differential properties. A theoretical picture will be proposed which postulates different post-synaptic sites and transmitter actions to explain the origins and properties of such post-synaptic potentials.

## METHODS

The rabbit superior cervical ganglion with its pre- and post-ganglionic nerve trunks was isolated (from rabbits under paraldehyde anaesthesia) and mounted in a chamber, as already described (Eccles, 1952*a*). The ganglion was freed from all external connective tissue, including the sheath, under a binocular microscope. Usually one or two small snips were made in the cleaned ganglion so that thorough penetration, particularly by Botulinum toxin, might be facilitated, although it is questionable whether this was necessary. The stimulating leads on the preganglionic trunk were arranged with the cathode proximal to the ganglion, with an earthing electrode between cathode and ganglion; the active recording electrode supported the ganglion usually at its cranial, post-ganglionic pole, the second lead being located some millimetres away, at or near the cut end of the post-ganglionic nerve. The Ringer–Krebs solution had the same composition (except for 1·0 mM $Mg^{2+}$, instead of 0·5 mM) as that used earlier (Eccles, 1955). All the drug effects studied (unless otherwise stated) were tested *in vitro* on these ganglion preparations, at 35–38° C. Adrenaline, however, was tested on the responses of ganglia *in vivo*, since the concentration of adrenaline in the bathing solution of an isolated preparation is subject to the vagaries of oxidation both enzymically and non-enzymically in the high $pO_2$ of the bath. All ganglionic responses were recorded on an oscilloscope after amplification by a DC amplifier.

The Botulinum toxin was a sample of Type A, P-3 received from Porton in 1954. A mouse assay showed the $LD_{50}$ to lie in the range $1–4 \times 10^{-6}$ mg, *i.e.* it was close to the original value given, $2 \times 10^{-6}$ mg (see Brooks, 1956). The toxin was weighed out and dissolved in 1 % $Na_2HPO_4$ with 0·2 % gelatine at a pH of 6·6. The dissolved toxin was kept at −20° C, there being no loss in toxicity over 3 weeks (Ambache, 1951). The amount of solution containing toxin or other drugs which was added to the environment of any ganglion was too small to cause any appreciable dilution of the Ringer's solution. The slight increase in phosphate that did occur with addition of toxin was shown to be ineffective, as responses of a ganglion in a solution at equivalent phosphate level were unaltered over 5 hr. The usual precautions were taken when handling Botulinum toxin. Any solutions that contained or could have been contaminated by toxin were boiled for 30 min before they were discarded.

To produce curarization of the ganglia dihydro-$\beta$-erythroidine hydrobromide was applied in a concentration (usually about 10 $\mu$g/ml.) which was just sufficient to extinguish the spike discharge of the ganglion even upon repetition (although if a very small spike appeared only among the first few responses of a tetanic train it was tolerated).

## RESULTS

### *Preganglionic control*

*Preganglionic fibre type.* The preganglionic fibres supplying the rabbit's superior cervical ganglion may be classified into the lower threshold, myelinated, B group and the higher threshold, unmyelinated, C fibres (Bishop & Heinbecker, 1932). It had been reported earlier (Eccles, 1952*a*) that stimulation of fibres having conduction velocities of the first group initiates the $S_a$ spike discharge in the uncurarized ganglion. Stronger stimuli, which also excite the fibres having conduction velocities of the second group, added a later $S_b$ spike discharge to the ganglion response, and also produced a considerable enlargement in the after-positivity that follows the response to a single volley. These findings were confirmed in three ganglia of the present series. A similar correlation of post-ganglionic spike discharges with B and C fibre preganglionics was found for the turtle's superior cervical ganglion by Laporte & Lorente de Nó (1950*a*). It should be noted that our preganglionic recordings did not permit clear observation of the appearance of the slower subgroups of the B fibres, described by Bishop & Heinbecker (1932). Any special significance that such fibres might have for the initiation of the various responses investigated in this work would therefore remain unknown. Also, the maximum height of the $S_a$ spike usually was achieved only at a stimulus strength which was above threshold for the $S_b$ response (e.g. Fig. 4*E*), making it difficult to exclude the possibility that some of the lower threshold fibres of the C group might be contributing to the later part of the $S_a$ spike discharge.

In the present work, the strength of the stimulus required for the excitation of B and C preganglionic fibres was studied in relation to production of the P and LN potentials by tetanic trains of preganglionic impulses (usually 40/sec, with 0·5 or 1 sec train duration) in just-curarized ganglia. In most experiments the preganglionic action potentials were not actually monitored; instead, the stimulus strengths required to produce the maximal $S_a$ and $S_b$ spike discharges of the uncurarized ganglion were used as an approximate index of the activation in that preparation of the B group and of the B plus C groups respectively (e.g. Figs. 1, *IA*, *E* and 4, *E*, *A*). These stimulus strengths will be referred to as maximum $S_a$ and maximum $S_b$ respectively.

In most cases, the P wave of the curarized ganglion showed a definite and often large increase when preganglionic stimulus strength was raised from max. $S_a$ to max. $S_b$ (e.g. Figs. 4*N*, *R*; 5*G*, *J*; and 1, *IIC*, *G*, *D*, *H* in which $S_a$ alone, in *C* and *D*, was slightly submaximal). Laporte & Lorente de Nó (1950*b*) also demonstrated a large production of P potential by C fibre preganglionic volleys in curarized turtle ganglia. With a stimulus

strength of about one half to two thirds max. $S_a$, the P wave was correspondingly lower (e.g. Fig. 4*J*, *N*). In four out of ten ganglia, however, the changes in P wave were small, or negligible, or somewhat variable (e.g. Fig. 3*I*, *M*). Since P was significantly larger at the max. $S_b$ strength, the latter was included in all the test situations reported in this paper.

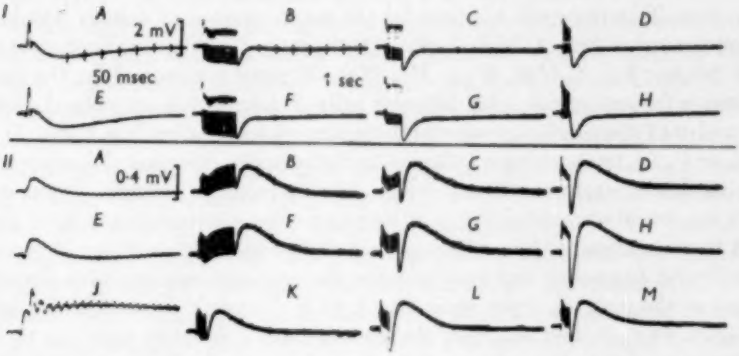



Fig. 1. Effects of stimulus intensity, frequency, and train duration (total number) of preganglionic volleys on ganglion responses. Section *I A–I H*, before curarization; section *II A–II M*, after curarization with dihydro-β-erythroidine-HBr, 7·5 μg/ml. For both horizontal rows *I A–D* and *II A–D*, stimulus strength = 6 V, in this case somewhat submaximal for $S_a$ spike and below $S_b$ spike threshold; for both horizontal rows *E–H* and for row *J–M*, stimulus strength = 30 *V* at max. $S_b$ strength. Vertical column under *A*, single volley (except in *II J*, 40/sec); column under *B*, 10/sec train of stimuli for 2 sec duration (except *II K*, 10/sec for 0·5 sec); column under *C*, 20/sec for 1 sec (except *II L*, 20/sec for 0·5 sec); under D, 40/sec for 0·5 sec (except *II M*, 15/sec for 0·5 sec). Time scale in *I A* applies to vertical column under *A*; that in *I B* applies to all the rest. Voltage scale in *I A* applies to all of section *I*; that in *II A* to all of section *II*. Faint spike potentials have been retouched.

The LN wave, on the other hand, was either slightly or not at all greater when stimulus strength was raised from max. $S_a$ to max. $S_b$ (e.g. Figs. 1, *II C*, *G*, *D*, *H*; 3*I*, *M*; 4*N*, *R*; 5*G*, *J*). The magnitude of LN wave was also less sensitive than the P wave to a reduction of stimulus strength to about one half max. $S_a$ (e.g. Fig. 4*J*, *N*).

*Frequency and total number of preganglionic volleys.* It seemed possible that another way to differentiate the origins of the various components of the synaptic potentials might lie in examining their generation by different frequencies of preganglionic volleys. Since P and LN waves develop slowly and have long durations, the train duration or total number of preganglionic volleys might also have an effect, independent of the frequency. Consequently, tests were done (*a*) with different frequencies but with a constant number of twenty volleys, by suitably adjusting the

train duration at each frequency, and (*b*) with different frequencies at a constant train duration (i.e. varying total number of volleys as well as frequency).

In Fig. 1, *II*, it may be seen that the P wave which follows the preganglionic tetanus in a curarized ganglion shows a definite increase in amplitude and duration with increase in frequency of the preganglionic volleys. This occurred whether (*a*) the total number of volleys was kept constant at 20 (Fig. 1, *II F, G, H*) or (*b*) the train duration was kept constant at 0·5 sec (Fig. 1, *II K, M, L, H*). If the P wave is observed for the same tetanic frequency but with different train durations (i.e. varying the total number of preganglionic volleys) there is much less change (e.g. Fig. 1, *II F, K*, or *G, L*), or sometimes a change in the opposite direction (i.e. a larger P with shorter train duration). While only frequencies between 10/sec. and 40/sec. are illustrated in Fig. 1, P was seen to be even smaller at 6/sec than at 10 and somewhat larger (though not always so) at 60 or 80/sec. A change in P with frequency was seen whether the preganglionic stimulus strength was at the max. $S_a$ level, as in Fig. 1, *II B, C* and *D*, or at max. $S_b$, as in Fig. 1, *II F, G*, and *H*. Thus the post-tetanic P wave is sensitive to the frequency rather than the total number of the preganglionic volleys which elicit it, becoming maximal with about 40–60/sec tetani. In the uncurarized ganglion the after-positivity, presumably largely or entirely due in this case to true positive after-potentials, appeared to show less striking changes with change in frequency (Fig. 1, *I*).

The LN wave, which lasts for several seconds after the P wave in a curarized ganglion, appears to behave differently from the P wave. In condition (*a*), total number of volleys constant at 20, there was relatively little effect of change in frequency between 10/sec and 40 (or even 80)/sec (Fig. 1, *II B, C, D, F, G, H*). This result obtained at both strengths of preganglionic stimulus. Though not included in the figure, the LN wave after a 2/sec tetanus (train duration, 10 sec) was distinctly present but considerably smaller than at 6/sec (train duration, 3·2 sec). The LN wave achieved its maximal amplitude at about 6 or 10/sec, though its duration and total area tended to increase somewhat with further increase in frequency. On the other hand, in condition (*b*), with train duration fixed at 0·5 sec, the LN wave did increase progressively with increasing frequency at least up to 60 or 80/sec (e.g. Fig. 1, *II K, M, L, H*). This increase, however, can be explained by the increase in total number of volleys which occurred as the frequency was raised. This explanation is confirmed by observing the LN wave at a given frequency but with changing train duration (i.e. change in total number of volleys only), as in Fig. 1, *II K* and *F*, or *L* and *G* (see also Eccles, 1952*b*). Thus the LN wave increases significantly in amplitude and duration mainly with increase in total

number of volleys (at least between 2 and 20), and shows relatively little change with increase in frequency *per se* above 6 or 10/sec.

The above description has dealt only with the post-tetanically observed P and LN waves, and not with any responses during the tetanus. It has also ignored the masking of one potential by another simultaneously occurring one. If one examines the onset of the positivity and of the secondary negative swing during the repetitive train of stimuli in curarized ganglia, one sees that the swing toward positivity begins early in the tetanus, superimposed upon the N potential (Fig. 1, *II*; see also, Laporte & Lorente de Nó, 1950*b*; Eccles, 1952*b*). The secondary negativity, however, does not become evident until about 0·4 sec of the tetanus has elapsed. It seems likely that the secondary negative swing in the base line that may be seen during a tetanus (e.g. Fig. 1, *II B, C, F, G*) is part of the same process that leads into the LN wave which is seen following the tetanus (see also Eccles, 1952*b*, Figs. 3, 9, 10). When the P wave has been almost completely abolished by dibenamine (see below), the remaining partially depressed post-tetanic LN wave becomes evident immediately after the 0·5 sec tetanus is over (Fig. 5 *K, L*). Even if this secondary negative base-line swing *during* a tetanus were to be included in the total LN potential, it would not appear to modify seriously the conclusions derived with respect to changes in LN potential under the conditions tested.

Electrical interaction by the overlapping of N, P and LN during a tetanus make it impossible quantitatively to determine the values of each response. The estimate of the component of P potential during the tetanus would seem to be more obscure than that of N or LN, even for a train lasting 0·5 sec or less so as to avoid the onset of the late negativity during stimulation (Fig. 1, *II K, M, L, H*). Thus one is less confident than in the case of the LN response about the influence of any changes in P potential during a tetanus on conclusions derived from observing only the post-tetanic component of P. However, under conditions in which the N potential is relatively suppressed (heavy curarization), the P wave recorded during a 20/sec tetanus appeared to show relatively little increase in amplitude as the train duration was increased from 0·2 to 3·0 sec, though there was some increase in the duration, i.e. total area, of the P wave (Eccles, 1952*b*, Fig. 6).

The overlapping of the P and LN components must be borne in mind when assessing even their post-tetanic values. For example, the marked reduction in amplitude of the LN wave by the addition of an anti-cholinesterase to a heavily curarized ganglion is coupled with an increase in the amplitude but especially in the duration of the P wave (Eccles, 1952*b*).

*Botulinum toxin*

After introducing Botulinum toxin into the solution bathing an uncurarized ganglion, the ganglionic responses to preganglionic volleys were tested periodically. These volleys were not continuous, being delivered in testing groups at intervals of about $\frac{1}{2}$–1 hr. In all experiments except the first a concentration of 20,000 mouse $LD_{50}$ units of toxin per millilitre of bath fluid was used. With this dose of toxin it was found that the spike responses to single volleys progressively decreased to zero within 2–3 hr (e.g. Fig. 2*A*, *B*, *C*, *D*), leaving behind a synaptic potential which also gradually disappeared within another hour or two (Fig. 2*D*, *E*, *F*, *G*). Repetitive stimulation could still elicit either spikes or negativity for some time after single volleys were no longer effective (e.g. Fig. 2, bottom three horizontal rows). At no time during this blocking action of the toxin could any change be detected in the stimulus thresholds or action potentials of the preganglionic B or C fibre groups.

As in curarized ganglia, a post-tetanic P wave was visible (after a 40/sec tetanus lasting 1 sec, Fig. 2 *W*, *X*, *Y*) even when there was no longer any evidence of spike discharges (Fig. 2, horizontal rows *E*, *L*, *S*; *F*, *M*, *T*; and *G*, *N*, *U*). In contrast with the curarized ganglion, an LN wave was very small (Fig. 2 *W*) or absent during the Botulinum depression. Furthermore, there appeared to be no obvious differential blocking of the various potential components; the N and P waves (and LN, when present) decreased together progressively during the action of the toxin (Fig. 2 *W*, *X*, *Y*).

Several other points of interest may be noted in Fig. 2. (*a*) The $S_a$ and $S_b$ spikes did not disappear at similar times of Botulinum action, the $S_b$ spike outlasting the former (Fig. 2*C*, *D*, vs. *A*, *B*). (*b*) Even when the ganglion spike has been greatly reduced, as tested with single preganglionic volleys (Fig. 2*C*, *D*), it was increased and sustained upon repetitive activation at 40 and 80/sec (Fig. 2*J*, *K*, *Q*, *R*). (*c*) The same applied even more strikingly to the negative synaptic potential, N, in the absence of spikes. Upon repetition at 40/sec, the N response rapidly increased several fold and tended to continue increasing during the course of the 0·2 sec train, even when the single synaptic response was almost gone (Fig. 2*E*, *F*, *G*, and *L*, *M*, *N*). At 80/sec (Fig. 2*S*, *T*, *U*) the N response was considerably greater than at 40/sec, especially in the late stages of blocking (Fig. 2*T*, *U*, vs. *M*, *N*). Of further interest was the finding that after the toxin had suppressed the spike responses, dihydro-β-erythroidine, (12·5μg/ml.), produced a further rapid decline of all the remaining ganglion responses. Eserine (1μg/ml.) added at a similar stage to a toxin-treated ganglion had little or no effect on the course of events; there was

a questionable small increase in duration, and perhaps in amplitude, of the P wave coupled with a loss of what appeared to be a remnant of an LN potential.

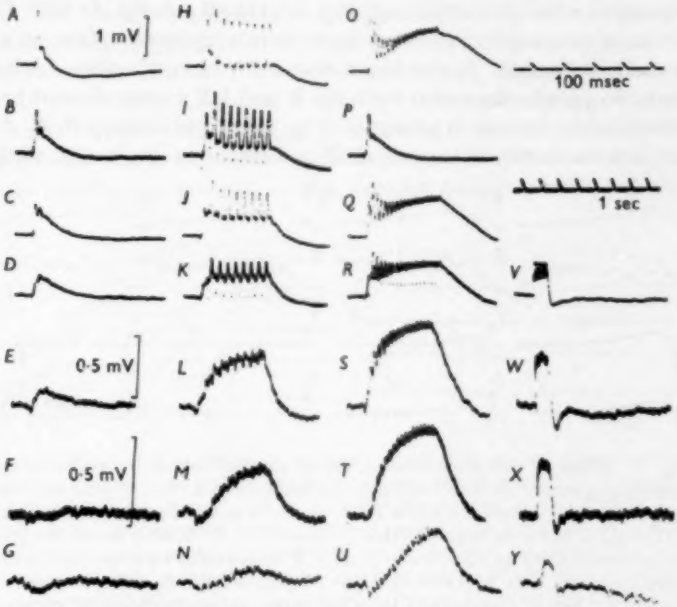



Fig. 2. Progressive effects of Botulinum toxin. Horizontal row, *A*, *H*, *O*, responses before toxin; row *B*, *I*, *P*, 1 hr 25 min after addition of 20,000 mouse $LD_{50}$ u. Botulinum toxin/ml. bath fluid; *C*, *J*, *Q*, 2 hr; *D*, *K*, *R*, *V*, 2 hr 30 min; *E*, *L*, *S*, *W*, 3 hr 15 min; *F*, *M*, *T*, *X*, 3 hr 50 min; *G*, *N*, *U*, *Y*, 4 hr 25 min. Vertical column *A–G* shows response to a single volley at each of the respective times. Vertical column *H–N*, responses to a brief 40/sec tetanus; column *O–U*, response to a brief 80/sec tetanus (except *P*, single volley at same stimulus strength as *H*, *I* and *O*); column *V–Y*, responses to a 1 sec train at 40/sec. Although all stimulus strengths were close to or above that required for producing the max. $S_a$ and $S_b$ spikes in the normal ganglion, they unfortunately were not identical and will therefore be listed by vertical column: (*A* 17·5 V; *B*, *C*, *D* 22·5; *E*, *F*, *G* 25; *H*, *I* 15; *J*, *K*, *L* 22·5; *M*, *N* 25; *O*, *P* 15; *Q*, *R*, *S* 22·5; *T*, *U* 25; *V*, *W* 22·5; *X*, *Y* 25. The voltage scale in *A* applies to the horizontal rows starting with *A*, *B*, *C*, and *D*; that in *E* applies to horizontal row *E*; that in *F* applies to the horizontal rows *F* and *G*. The time scale labelled 100 msec applies to the first three vertical columns (under *A*, *H* and *O*); the 1 sec scale applies to vertical column *V*, *W*, *X*, *Y*. Faint spikes have been retouched.

### *Atropine*

Atropine was employed in order to test the possibility that more than one type of cholinergic receptor site might be involved in the synaptic generation of the N, P and LN responses. It was added to the bathing

medium of ganglia which were already moderately curarized with dihydro-$\beta$-erythroidine (approximately 10$\mu$g/ml.) and which were thus clearly exhibiting the N, P and LN responses, uncomplicated by spike discharge or by positive after potentials (e.g. Fig. 3, vertical column *A*; with 40/sec repetition of preganglionic volleys there were very small spikes on a few of the early responses). It was found that a low concentration of atropine sulphate, 0·1$\mu$g/ml., depressed both the P and LN waves without having any effect on the tetanic N potential (Fig. 3, vertical column *B*, *F*, *J*, *N*). In fact, in some instances the tetanic N potential was larger with atropine.

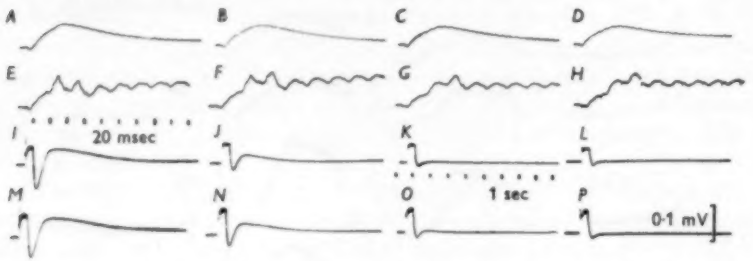



Fig. 3. Effect of atropine on responses of ganglion already curarized (with dihydro-$\beta$-erythroidine-HBr, 10$\mu$g/ml.). Vertical column *A*, *E*, *I*, *M*, after curarization but before atropine; column *B*, *F*, *J*, *N*, after adding atropine sulphate, 0·1$\mu$g/ml., to the curarizing bath fluid; column *C*, *G*, *K*, *O*, after raising atropine concentration to 0·5$\mu$g/ml.; column *D*, *H*, *L*, *P*, after raising atropine to 8·0$\mu$g/ml. Horizontal row *A–D*, responses to single volleys, at max. $S_b$ spike strength (as determined before curarization) for each respective concentration of atropine. Horizontal rows *E–H* and *M–P*, responses to 40/sec tetanus of 0·5 sec duration, at max. $S_b$ spike strength; row *I–L*, responses to similar tetanus, but at max. $S_a$ spike strength (just above threshold for the $S_b$ spike complex). Voltage scale same throughout. Time scale of 20 msec under *E* applies to horizontal rows *A–D* and *E–H*; 1 sec scale under *K* applies to rows *I–L* and *M–P*. Faint tracings have been retouched.

Such increases in N were probably due to the depression of a component of the P potential which may be occurring during the tetanus. The single-volley N responses were usually somewhat reduced by atropine; they were never increased (e.g. Fig. 3*A*, *B*, *C*, *D*). When atropine concentration was increased to 0·5 and 8·0$\mu$g/ml. (Fig. 3*C*, *G*, *K*, *O* and *D*, *H*, *L*, *P*, respectively), the LN wave was completely gone, but a small P wave resisted abolition even at the 8$\mu$g/ml. concentration. At the higher concentrations some depression of the tetanic N wave became noticeable in some experiments; but it is significant that a large increase, from 0·5 to 8·0$\mu$g/ml., for example, could occur with relatively little change in tetanic N (Fig. 3*K*, *O* vs. *L*, *P*).

*Dibenamine*

Dibenamine and several other drugs (see below) were utilized in an attempt to block any adrenergic step that might be involved in producing the slow synaptic potentials. Dibenamine was tested in the same way as atropine, i.e. on moderately curarized ganglia (e.g. Fig. 4, vertical column *B*, *F*, *J*, *N*, and Fig. 5, column *A*, *D*, *G*, *J*). A longer soaking time, at least 45–60 min instead of the usual 20–30 min, was permitted with dibenamine, since its action following intravenous injections *in vivo* takes considerable time to develop; a test run on the isolated ganglion indicated that the

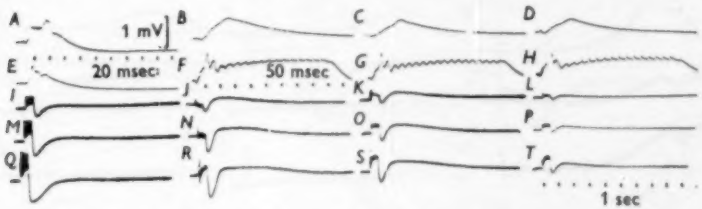



Fig. 4. Effect of dibenamine on responses of ganglion already curarized (with dihydro-β-erythroidine-HBr, 7·5 μg/ml.). Vertical column *A*, *E*, *I*, *M*, *Q*, ganglion before curarization; column *B*, *F*, *J*, *N*, *R*, after curarization; column *C*, *G*, *K*, *O*, *S*, after adding of dibenamine 1 μg/ml. to the curarizing bath fluid; column *D*, *H*, *L*, *P*, *T*, after raising dibenamine concentration to 4 μg/ml. Horizontal row *A–D*, responses to single volleys and row *F–H*, to brief tetanus at 40/sec, all at max. $S_b$ spike strength; *E*, single volley at max. $S_a$ spike strength (slightly above threshold for $S_b$ spike complex; compare with *A*). Horizontal row *I–L*, responses to 0·5 sec tetanus at 40/sec, with stimulus strength somewhat submaximal for $S_a$ spike, i.e. below that in *E*; row *M–P*, similar to *I–L* but with max. $S_a$ stimulus strength, as in *E*; row *Q–T* similar to *I–L* but with max. $S_b$ stimulus strength, as in *A*. Voltage scale same throughout. Time scale under *A* for *A*, *B*, *C*, *D*, *E*; scale under *F* for *F–H*; scale under *T* for *I–T*. Over-exposed, thickened tracing in *K* was retouched.

maximal effect was achieved after this longer soaking period. At a concentration of 1 μg/ml. there was a strong depression of the P wave, a smaller depression of at least the total area under the LN wave, and no depression or an increase in the tetanic N potential (e.g. Fig. 4, column *C*, *G*, *K*, *O*, *S*), although the single volley N wave was reduced (Fig. 4*C*). At higher concentrations (Fig. 4, columns *D*, *H*, *L*, *P*, *T*; and Fig. 5, columns *B*, *E*, *H*, *K* and *C*, *F*, *I*, *L*) there was a reduction in the tetanic N wave as well as further decreases in the sizes of the P and LN waves (Figs. 4*K–L*, *O–P*, and *S–T*; and 5*G–I* and *J–L*), and in the single N response (Figs. 4*C–D*, and 5*A–C* and *D–F*). There would seem to be a less differential specificity for the dibenamine depression of the P and LN waves as opposed to the N potential than is the case for atropine. However, it seems clear

that the P wave is distinctly more sensitive to dibenamine than is LN, and the LN considerably more so than the N potential.

*Adrenaline inhibition and dibenamine.* When injected into the blood supply of a ganglion with its circulation intact (Marazzi, 1939*b*; Lundberg, 1952), adrenaline reversibly depresses the ganglion spike responses, as is illustrated in Fig. 6*A*, *D*, *G*. There was no evidence of facilitation with these single test volleys. This confirms earlier reports in which similar dosages were used (Lundberg, 1952).

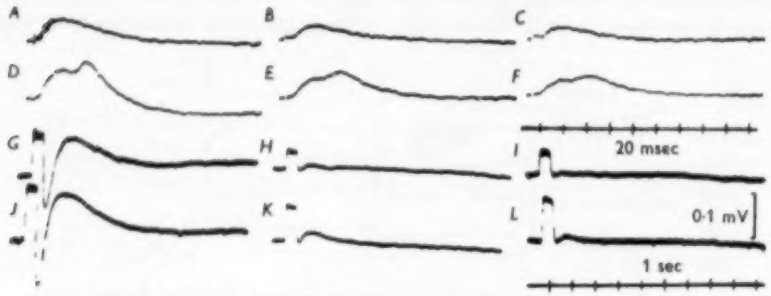



Fig. 5. Effect of dibenamine in a higher range of concentrations on responses of curarized ganglion. Vertical column *A*, *D*, *G*, *J*, after curarization (dihydro-β-erythroidine-HBr, 7·5 μg/ml.) but before dibenamine; column *B*, *E*, *H*, *K*, after adding dibenamine, 5 μg/ml. to curarizing bath fluid; column *C*, *F*, *I*, *L*, after raising dibenamine concentration to 8 μg/ml. Horizontal row *A*–*C*, responses to single volleys, and row *G*–*I*, to 0·5 sec tetanus at 40/sec, both rows at stimulus strength for max. $S_a$ spike (as determined in this ganglion before curarization); rows *D*–*F* and *J*–*L* similar to *A*–*C* and *G*–*I* respectively, but at stimulus strength for max. $S_a$ plus $S_b$ spike. Voltage scale same throughout. Time scale under *F* for *A*–*F*; scale under *L* for *G*–*L*.

If repetitive volleys at 40/sec and 1·0 sec train duration were used as tests, two phases of depression and facilitation of the spike were seen (Fig. 6*E*): (*a*) The initial spike was depressed as in Fig. 6*D*, but the next few spikes were in the range of amplitude seen without adrenaline. (Total spike height should be measured from the base line prevailing during, not before, the tetanus, if these are to be compared for different resting trains, since there is also a change in the after-positivity, Fig. 6*A*, *D*). This large facilitation of a second spike, following the first by about 25 msec, was already reported by Lundberg (1952). (*b*) However, following these few facilitated spikes the amplitude again dropped off by about 15 or 20%, and gradually built up once more towards the normal height over a period of about half a second. An initial depression followed by some facilitation and then by a second period of depression of transmission of a train of volleys may also be seen during a tetanus in partially curarized ganglia

(e.g. Eccles, 1952*b*); but the subsequent second recovery or facilitation phenomenon during a tetanus, in a ganglion subjected to adrenaline, is not seen with partial curarization.

Some hours after the end of a slow intravenous injection of *dibenamine*, about 25 mg/kg, the adrenaline test was repeated (Fig. 6*C*, *F*, *I*). There was now much less reduction in spike height (both $S_a$ and $S_b$ components)

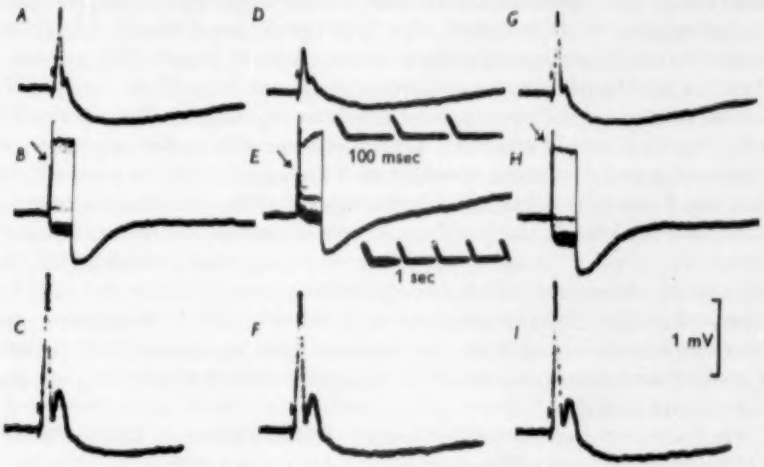



Fig. 6. Effect of adrenaline, before and after dibenamine, on ganglion responses. *In vivo* preparation, rabbit (approx. 2 kg) anaesthetized with paraldehyde. Vertical column *A–C*, responses at the start of, or just before, the injection of adrenaline; column *D–F*, at approximately the time of the peak effect following an intravenous injection of 15 $\mu$g of adrenaline bitartrate (*D* at 24 sec, *E* at 30 sec, *F* at 24 sec after injection); column *G–I*, still later (*G* and *H* at 120 sec, *I* at 50 sec after injection). Horizontal row *A*, *D*, *G*, responses to single volleys, max. for $S_a$ and somewhat submaximal for $S_b$ spike strength, delivered at a continuous rate of 1/1·7 sec during the adrenaline test; line *B*, *E*, *H*, responses to 1 sec tetani at 40/sec, delivered periodically at about 10–12 sec intervals during the first minute after injecting adrenaline and then at 30 sec intervals (arrows point to the tip of the first spike of each tetanus shown); row *C*, *F*, *I*, as in line *A*, *D*, *E*, but taken approximately 3 hr 20 min after the end of a very slow intravenous injection of about 50 mg dibenamine-HCl. Voltage scale same throughout. Time scale under *D* for rows *A*, *D*, *G* and *C*, *F*, *I*; scale under *E* for row *B*, *E*, *H*. Faint spikes have been retouched.

and in the after-positivity (compare with Fig. 6*A*, *D*, *G*). Lundberg (1952) has shown a similar antagonism of dihydroergotamine against the action of injected adrenaline. Since the full effect of dibenamine did not occur until about 3 hr after its injection, this may explain Matthews's failure (1956) to find an antagonism between adrenaline and dibenzyline, a close relative of dibenamine. That a blood level of dibenamine, sufficient to

antagonize most of the effects of injected adrenaline, did not itself completely suppress the P and LN potentials could be shown after curarization of such a preparation.

*Other drugs*

*Ergotamine (tartrate); dihydroergotamine (methane sulphonate).* Each of these drugs was tested on an isolated, curarized ganglion (one each) at concentrations of 1–20 $\mu$g/ml. and 2–20 $\mu$g/ml. respectively. At these concentrations there was little effect, if any, on the N, P and LN potentials.

*1-(3′,4′-dichlorophenyl)-2-isopropylaminoethanol-HCl.* This compound, the chloro-analogue of isoproterenol, has been reported by Slater & Powell (1957) to block certain inhibitory actions of adrenaline and of isoproterenol in smooth muscle, in concentrations of 2–100 $\mu$g/ml. On the supposition that the P wave in curarized ganglia represents an inhibitory action of adrenaline released in the ganglion, it was of interest to test the relative sensitivity of the P wave to this drug. The only result obtained was a non-specific depression of all the synaptic potentials (N, P and LN) in curarized ganglia, at concentrations of 5 and 10 $\mu$g/ml. All these responses were virtually abolished when the concentration was raised to 30 $\mu$g/ml. A similar depression was seen for the spike and after-positivity in the uncurarized ganglion.

*5-hydroxy-tryptamine (5-HT); lysergic acid diethylamide (LSD).* When 5-HT was added to an uncurarized ganglion in a concentration of 0·1 $\mu$g/ml., there was a slight reduction in the spike heights ($S_a$ and $S_b$) and in the after-positivity following a single volley or following a 40/sec tetanus lasting 1 sec. An increase in 5-HT concentration to 1·0 $\mu$g/ml. was followed by further slight reductions, $S_a$ more than $S_b$. Washing for about 2 hr in fresh Ringer's solution partially restored the losses in amplitude of spike and after-positivity. Thus 5-HT appeared to have a slight depressant action, in the same way as adrenaline; but since it was not tested in a perfused ganglion the quantitative estimation of its effectiveness is in doubt.

To help test the possibility that 5-HT might be a natural synaptic mediator in the origin of the P or LN waves, LSD was added to a curarized ganglion. LSD in very low concentrations (0·01 $\mu$g/ml.) has been shown to antagonize certain actions of 5-HT (Gaddum, 1957). When LSD was added, in a concentration of 0·01 or 0·1 $\mu$g/ml., to a ganglion curarized by dihydro-$\beta$-erythroidine, there appeared to be little or no change in P and in LN, relative to the N potential, which hardly changed. The drug was only tested in one preparation, but that was sufficient to show that its effectiveness was much less than that of atropine or dibenamine.

## DISCUSSION

*Post-synaptic nature of the N, P, and LN potentials.* Earlier work has already shown that the N response of the curarized ganglion appears to be identical with a depolarizing post-synaptic potential that is recorded in single ganglion cells by means of an intracellular micro-electrode (Eccles, 1955). Like the N response, the P and LN potentials in curarized ganglia occur in the absence of any ganglionic spike discharge and they should not, therefore, be thought of as ganglionic after-potentials (see also Laporte & Lorente de Nó, 1950*b*). This latter conclusion is further supported by the lack of any effect of veratrine (approx. $10^{-7}$ and $10^{-6}$M) on the N, P, or LN responses, even though this drug greatly increased the negative after-potential following spikes in uncurarized ganglia (R. M. Eccles, unpublished observations). The present work shows that the P and LN potentials of lightly curarized ganglia can be readily suppressed by low concentrations of other known post-synaptic blocking agents, namely atropine or dibenamine, with relatively little effect on the N potential. In addition, a recording from the preganglionic nerve, with one lead less than 1 mm away from the preganglionic pole of the ganglion, shows no appreciable N, P, or LN potentials when these are elicited in the ganglion (Libet, unpublished). The evidence at present thus strongly supports the view that the P and LN potentials, like the N potential, are also part of the post-synaptic response.

*Differential nature of the N, P, and LN potentials.* In the present work it is seen that the P and LN components of the potentials recorded in curarized ganglia are differently affected by the strength of the preganglionic volley, and by the frequency as well as by the total number of these volleys (Fig. 1). The striking sensitivity of both the P and LN potentials to suppression by low concentrations of atropine or dibenamine is in sharp contrast to that of the initial N potential (Figs. 3, 4, and 5); with these drugs one can retain a tetanic N potential that is unaffected or even greater than in the control, curarized state (though there may be some decrease of the single-volley N response), while virtually eliminating the P and LN potentials. There is also a discernible difference between P and LN sensitivities as well; P is the more sensitive to dibenamine, a small LN potential being retained even with P completely abolished (Figs. 4 and 5), while LN is the more sensitive to atropine (Fig. 3). Earlier work (Eccles, 1952*a*) had already shown other differential sensitivities to blocking agents. The N potential is much more sensitive to curariform blockers; with these drugs large P and LN potentials can be retained when the N potential has been almost entirely eliminated. Anti-cholinesterases such as prostigmine have a further differential effect on strongly curarized

ganglia, in that they can suppress the LN wave and leave behind a large P wave which then is present in a virtually pure form (Eccles, 1952*b*). All this evidence seems to leave little doubt that the N, P, and LN components of the response in the curarized ganglion are separate responses which probably have different post-synaptic origins.

*Theory to explain the origins of the various post-synaptic potentials*

It is already accepted that the spike response of the post-ganglionic unit to preganglionic activation, and the initial depolarizing (N) post-synaptic potential, is cholinergically mediated (see Eccles, 1957).

*The P potential.* The existence of chromaffin cells and their innervation by preganglionic nerve fibres in sympathetic ganglia has been described (for refs. see Bülbring, 1944, and Lundberg, 1952). Such cells appear to be generally associated with the content of adrenaline, rather than noradrenaline, in tissues other than the adrenal medulla (von Euler, 1959). A significant output of adrenaline from sympathetic ganglia following stimulation of the preganglionic nerve has in fact been reported (Lissák, 1939; Bülbring, 1944). In addition, Lundberg (1952) had reported the occurrence of some rise in the demarcation potential of sympathetic ganglia in many, though not all, instances following the intravenous injection of adrenaline. When this evidence is coupled with our finding that dibenamine can eliminate the P potential with relatively little effect on the N response, it adds up to strong support for the view that the P potential (in curarized ganglia) is elicited by an adrenaline-like substance. The latter would be secreted by the chromaffin cells in response to preganglionic volleys and, after diffusion to appropriate sites on the post-ganglionic neurone, would initiate a hyperpolarizing response (the surface-positive, P, wave) at the membrane of this cell. The latency of onset for P (or LN) is virtually impossible to estimate accurately because of the temporal interaction that is possible among the N, P and LN components. However, P latency appears to be much longer than that for N, and certainly its duration, with single or repetitive preganglionic volleys, is much longer (even seconds, in the presence of prostigmine). Such slow characteristics would fit with the proposed adrenaline secretion-diffusion mechanism.

We find that Botulinum toxin gradually suppresses all the ganglion responses without any definite distinctions. We may therefore assume that there are synaptic junctions that involve the release of acetylcholine which are interposed in the mechanisms originating all the post-synaptic components. In view of the proposed position for a transmitter action of adrenaline (see above), the interference with the P response by Botulinum toxin would have to be placed at a prior step in the sequence, i.e. at the

activation of the chromaffin cells by the preganglionic nerve endings (see Fig. 7). The cholinergic synapse leading to the P potential is relatively insensitive to curariform drugs (or to penta- and hexa-methoniums, R. M. Eccles, unpublished observations), but is readily suppressed by low concentrations of atropine, hence it would appear that the receptor sites for ACh on the chromaffin cells are different from N sites on the ganglion cell, i.e. they are perhaps muscarinic rather than nicotinic in nature. This part of the hypothesis could be tested by comparing the effects of atropine with curare- or nicotine-like blocking agents on the actual release of adrenaline-like substance following preganglionic nerve stimulation.

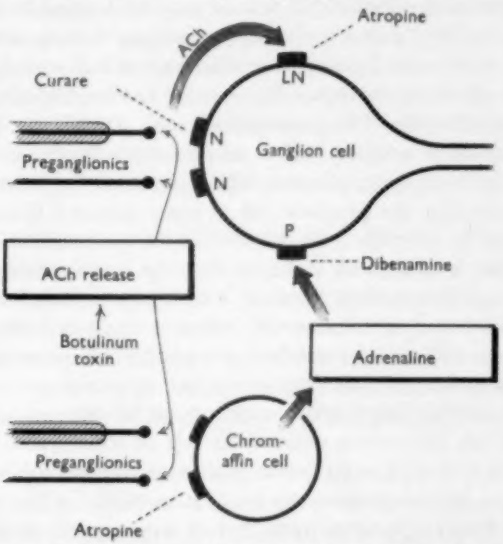



Fig. 7. Diagram of theory suggested to explain the synaptic origins and blockade of the N, P, and LN potentials. Receptor sites for the transmitter substances are shown as small blocks on cell membranes. (It should be noted that no significance should be attached to the particular locations of the P and LN sites with respect to either the axon hillock or the subsynaptic N receptor sites; the N, P and LN sites derive their location, as well as their number, on the ganglion cell membrane in the diagram from convenience in illustrating, not because these parameters are known.)

*The LN potential.* The results with Botulinum toxin indicate that this component also requires an ACh-release step. However, the insensitivity of the LN potential to blocking by curariform drugs (or by the penta- and hexa-methoniums, R. M. Eccles, unpublished observations), coupled with a great sensitivity to atropine, would indicate that the cholinergic receptor sites for this depolarizing post-synaptic response are also different from those mediating the N synaptic potential. (It may be recalled that the

penta- and hexa-methoniums are highly specific for nicotinic-type receptors, and do not have any atropinic or muscarinic actions; Paton & Zaimis, 1952.) This muscarinic, rather than nicotinic, type of susceptibility of the LN response is the chief reason for proposing separate receptor sites on the ganglion cells as the origin of the LN response. However, the effect of the anti-cholinesterases in suppressing the LN potential indicates that the bonding properties of such separate receptor sites for the LN response may be different from the known muscarinic as well as nicotinic ACh receptors.

Although an estimate of LN latency is subject to the difficulties already discussed, a probable value of 0·2–0·4 sec may be derived from the latency of onset of the late negative swing appearing during a tetanus (e.g. Fig. *I*, *II*, *B*, *C*, *F*, and *G*), and from the onset of LN in a single response in ganglia in which the recorded P potential has been abolished or made small in relation to the LN potential (Eccles, 1952*b*, see Figs. 13, 14). Such a long latency, coupled with a much longer duration, suggests that the different post-synaptic receptor sites postulated for mediating LN are either (*a*) located on the ganglion cell at some distance from the place of ACh release, or (*b*) that the ACh released at the preganglionic terminals is not immediately available for diffusion into the space outside the primary subsynaptic regions (perhaps because of slow release from the bonds with the N receptors and/or because of diffusion barriers). There is in the present work no evidence as to whether such LN receptors are present on all ganglion cells rather than only on special ones, nor any evidence as to their location on the ganglion cell with respect to the various parts of the neurone.

The proposed mechanisms have been summarized by a diagram in Fig. 7. Preganglionic impulses arriving at endings on the ganglion cells release ACh. This reacts with immediately subsynaptic sites on the post-synaptic membrane to elicit the primary depolarizing synaptic response, the N potential; these sites are readily blocked by curariform agents but not by atropine in low concentrations. When this ACh diffuses away from the primary subsynaptic regions either before or after temporary binding with the N receptor sites, it may encounter and become bound to LN receptor sites, located on this or perhaps on adjacent ganglion cells, with the consequence of a slowly arising and long-lasting depolarization, the LN potential. The binding of ACh at LN receptor sites is relatively quite insensitive to curariform agents (in fact these seem to facilitate the LN response) but it is highly sensitive to blocking by atropine. Other preganglionic terminals at the chromaffin cells also release ACh. In contrast to the N receptor sites on the ganglion cells, the post-synaptic sites on the chromaffin cells are very sensitive to atropine block, rather than to

curariform agents. Upon adequate excitation by the ACh released, the chromaffin cell 'secretes' adrenaline. The diffusing adrenaline encounters 'P' receptor sites on ganglion cells in the vicinity. This interaction results in changes tending to hyperpolarize the ganglion cell membrane, producing the P potential. The action of adrenaline at the P receptor sites is prevented by dibenamine.

The depression of the LN potential by low concentrations of dibenamine, although relatively less pronounced than the effect on the P potential, remains a difficult point. Additional postulates could be made to explain this result, but would require further experimental evidence before being proposed. The negative results with the other anti-adrenaline drugs tested for effects on the P response present less difficulty. The relative lack of specificity of the ergot drugs and the variable actions of some sympatholytic drugs in different types of tissues or situations is well known (Goodman & Gilman, 1955). Consequently, a positive result with the more specific adrenergic-blocker dibenamine would seem to be more significant than negative results with other drugs in the group.

*Possible pharmacological significance of P and LN responses*

Pharmacologically the P and LN response mechanisms could be very important apart from the question of their physiological role, the analysis of which must await further evidence. Since both the P and LN responses possess sensitivities to blocking agents which are quite different from those of the N synaptic potential, it is quite conceivable that they may also be uniquely activated by different drugs. The possible relationship of adrenaline-like compounds to the mechanism generating the P potential (and perhaps inhibition) has been discussed above. It is also possible that some drugs mimic the action of ACh at the postulated LN receptor sites in preference to the subsynaptic N receptor sites on ganglion cells; or they might mimic the action of ACh on the chromaffin cells, thereby affecting the release of adrenaline by those cells. One would predict from the present analysis that the effects of such compounds could be blocked by atropine, rather than by curariform or nicotinic drugs. A number of reports already in the pharmacological literature lend themselves to such an interpretation, e.g. the reports that the excitatory effect of pilocarpine on sympathetic ganglia is prevented by atropine rather than by curare or hexamethonium (Marazzi, 1939*a*; Trendelenburg, 1955). In any case it would seem profitable to evaluate and test the mechanisms for the effects of various drugs on sympathetic ganglia in the light of the differential properties of the various synaptic potentials which have been examined in the present paper.

SUMMARY

1. The characteristics of the slow positive (P) and late negative (LN) ganglionic potentials, which are prominent following tetanic stimulation of the preganglionic nerve of the curarized superior cervical ganglion, have been investigated. Different optima of thresholds, frequencies, and train durations were obtained for these potentials.

2. Botulinum toxin progressively depressed not only the post-synaptic spike response but also the N, P, and LN synaptic potentials.

3. Atropine in low concentrations could abolish the LN potential and strongly depress the P potential of curarized ganglia, with little effect on the N potential, i.e. the primary synaptic potential.

4. Dibenamine could strongly depress the P potential and less strongly the LN wave, with no depression or an increase in the tetanic N potential and a relatively small depression of the single-volley N responses.

5. The effect of an intravenous injection of adrenaline on ganglionic potentials was almost completely abolished by administration of dibenamine several hours earlier.

6. Little or no effects were obtained from other drugs—ergotamine, di-hydroergotamine, LSD, and 5-HT. The chloro-analogue of isoproterenol produced a strong but non-specific depression of the N, P, and LN potentials.

7. An hypothesis describing the synaptic origins of the ganglionic responses is developed in order to relate the various pharmacological actions to the events produced by the acetylcholine liberated from the preganglionic terminals.

We wish to express our thanks to Professor Sir John Eccles for his encouragement and for his helpful criticism of the manuscript; to Mr I. Keppie, the Ministry of Supply, Porton, for the sample of Botulinum toxin; to Sandoz for the ergotamine tartrate, dihydroergotamine methane sulphonate, lysergic acid diethylamide (LSD), and 5-hydroxytryptamine (5-HT); to Merck for our supply of dihydro-$\beta$-erythroidine hydrobromide; to Smith, Kline and French Laboratories for dibenamine; and to Dr I. Slater of Lilly Laboratories for the sample of 1-(3′,4′-dichlorophenyl)-2-isopropylaminoethanol-HCl.

The portion of this work done in Canberra was supported in part by a grant of a Commonwealth Fund Fellowship to one of us (B. L.). The portion of the work carried out at the University of California was supported by a grant (B–884) from the National Institute of Neurological Diseases and Blindness, and by one from the Breon Fund, administered by the Committee on Medical Research of University of California School of Medicine.

REFERENCES

AMBACHE, N. (1949). The peripheral action of *Clostridium Botulinum* toxin. *J. Physiol.* **108**, 127–141.

AMBACHE, N. (1951). A further survey of the action of *Clostridium Botulinum* toxin upon different types of autonomic nerve fibres. *J. Physiol.* **113**, 1–17.

BISHOP, G. H. & HEINBECKER, P. (1932). A functional analysis of the cervical sympathetic nerve supply to the eye. *Amer. J. Physiol.* **100**, 519–532.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of Botulinum toxin on miniature end plate potentials. *J. Physiol.* **134**, 264–277.

BÜLBRING, E. (1944). Action of adrenaline on transmission in the superior cervical ganglion. *J. Physiol.* **103**, 55–67.

BURGEN, A. S. V., DICKENS, F. & ZATMAN, L. J. (1949). The action of Botulinum toxin on the neuro-muscular junction. *J. Physiol.* **109**, 10–24.

ECCLES, J. C. (1957). *The Physiology of Nerve Cells*, pp. 182–184. Baltimore: The Johns Hopkins Press.

ECCLES, R. M. (1952*a*). Action potentials of isolated mammalian sympathetic ganglia. *J. Physiol.* **117**, 181–195.

ECCLES, R. M. (1952*b*). Responses of isolated curarized sympathetic ganglia. *J. Physiol.* **117**, 196–271.

ECCLES, R. M. (1955). Intracellular potentials recorded from mammalian sympathetic ganglion. *J. Physiol.* **130**, 572–584.

GADDUM, J. H. (1957). Serotonin-LSD interactions. *Ann. N.Y. Acad. Sci.* **66**, 643–648.

GOFFART, M. (1957). Action de l'adrénaline, de la noradrénaline et de l'isopropylnor-adrénaline sur la transmission synaptique dans les ganglions du système nerveux autonome. In *Colloques Nationaux du Centre National de la Recherche Scientifique*: L'adrénaline et la noradrénaline dans la régulation des fonctions homéostasiques. Paris: Centre National de la Recherche Scientifique.

GOODMAN, L. S. & GILMAN, A. (1955). *The Pharmacological Basis of Therapeutics*, 2nd ed., p. 579. New York: MacMillan.

LAPORTE, Y. & LORENTE DE NÓ, R. (1950*a*). Properties of sympathetic B ganglion cells. *J. cell. comp. Physiol.* **35**, Suppl. 2, 41–60.

LAPORTE, Y. & LORENTE DE NÓ, R. (1950*b*). Potential changes evoked in a curarized sympathetic ganglion by presynaptic volleys of impulses. *J. cell. comp. Physiol.* **35**, Suppl. 2, 61–106.

LISSÁK, K. (1939). Liberation of acetylcholine and adrenaline by stimulating isolated nerves. *Amer. J. Physiol.* **127**, 263–271.

LUNDBERG, A. (1952). Adrenaline and transmission in the sympathetic ganglion of the cat. *Acta physiol. scand.* **26**, 252–263.

MARAZZI, A. S. (1939*a*). Electrical studies on the pharmacology of autonomic synapses. I. Action of parasympathomimetic drugs on sympathetic ganglia. *J. Pharmacol.* **65**, 18–35.

MARAZZI, A. S. (1939*b*). Electrical studies on the pharmacology of autonomic synapses. II. The action of a sympathomimetic drug (epinephrine) on sympathetic ganglia. *J. Pharmacol.* **65**, 395–404.

MATTHEWS, R. J. (1956). The effect of epinephrine, levarterenol, and DL-isoproterenol on transmission in the superior cervical ganglion. *J. Pharmacol.* **116**, 433–443.

PATON, W. D. M. & ZAIMIS, E. J. (1952). The methonium compounds. *Pharmacol. Rev.* **4**, 219–253.

SLATER, I. H. & POWELL, C. E. (1957). Blockade of adrenergic inhibitory sites by 1-(3′, 4′-dichlorophenyl)-2-isopropylaminoethanol hydrochloride. *Fed. Proc.* **16**, 336.

TRENDELENBURG, U. (1955). The potentiation of ganglionic transmission by histamine and pilocarpine. *J. Physiol.* **129**, 337–351.

VON EULER, U. S. (1959). Autonomic neuroeffector transmission. In *Handbook of Physiology*, ed. FIELD, J. and HALL, V. Sect. 1: Neurophysiology, vol. I, ed. MAGOUN, H. W. Washington: American Physiological Society.

WINBURY, M. M. (1959). Mechanism of the local vascular actions of 1, 1-dimethyl-4-phenyl-piperazinum (DMPP), a potent ganglionic stimulant. *J. Physiol.* **147**, 1–13.

*J. Physiol.* (1961), **157**, *pp.* 504–512
*With 4 text-figures*
*Printed in Great Britain*

# THE MEASUREMENT OF METABOLIC AND VASCULAR RESPONSES IN THE THYROID GLAND WITH OBSERVATIONS ON ITS RESPONSES TO INSULIN, GLUCOSE AND ADRENALINE

BY F. O. DOSEKUN

*From the Department of Physiology, University College, Ibadan, Nigeria*

(*Received* 25 *January* 1961)

Grayson & Kinnear (1958) have extended the technique of internal calorimetry (Grayson, 1952; Carlyle & Grayson, 1956) to investigate problems of blood flow and metabolic heat production in solid organs. Dosekun, Grayson & Mendel (1960) have reported further investigations on human liver and rat skeletal muscle in which the validity of the technique of internal calorimetry in the determination of metabolic heat production was established.

The purpose of the present work was to apply this technique to the investigation of blood flow and heat production in the thyroid gland with particular reference to its carbohydrate metabolism.

## METHODS

The technique of internal calorimetry has been previously described (Grayson, 1952; Dosekun *et al.* 1960). It depends on the thermo-electric measurement of thermal conductivity by means of a heated thermocouple, to the heater circuit of which current is supplied in cyclic fashion as follows (Grayson & Mendel, 1957): 0·200 $mA^2$ for 24 sec, 0·600 $mA^2$ for 17 sec, 0·315 $mA^2$ for 24 sec, no current for 17 sec. This cycle is continuously repeated by means of a cam-operated switch. The temperature changes induced in the thermocouple by this procedure were recorded using a Cambridge D'Arsonval galvanometer, a light source and a recording camera. In all experiments the 'cold junction' was in the aorta. The calculation of thermal conductivity ($\delta k$) from the record has been fully described elsewhere (Dosekun *et al.* 1960). It has been shown previously that the difference between the thermal conductivity of living and dead tissue is a linear function of blood flow and in these experiments the function $\delta k$ has been taken to represent blood flow.

*Calculation of metabolic heat production.* The temperatures of a heated thermocouple through which currents of 0·200 $mA^2$ and 0·315 $mA^2$ are passing are called lower equilibrium and upper equilibrium temperatures respectively. As already reported (Dosekun *et al.* 1960), the lower equilibrium temperature could be measured from the bottom of the paper to the lower point of inflexion on the photographic trace. Changes in lower equilibrium temperature may be brought about by alterations in blood flow or by alterations in the temperature of the afferent blood; they may be brought about by alterations in local conditions of heat loss or they may be the result of changes in local heat production.

In the present experiments the 'cold junction' was situated in the aorta and changes in blood temperature were thus automatically compensated. The experiments were carried out

under conditions of constant temperature and humidity and changes in heat loss were a negligible factor. Alterations in lower equilibrium temperature were, therefore, due to a combination of blood-flow change and local heat production change. The calculation of thermal conductivity, however, allows a precise prediction to be made of the effect of blood-flow change alone on the lower equilibrium temperature. The difference between the actual recorded change and the change which ought to have occurred on the basis of this calculation has been designated 'excess temperature' and, as has been shown previously, may be used as an index of local heat production.

*Operative procedure.* The animals used in this work were male and female common Nigerian domesticated dogs (*Canis familiaris*) weighing between 7 and 11 kg. Anaesthesia was induced by intraperitoneal injection of pentobarbitone sodium (Nembutal, Abbott Laboratories) 0·5 ml./kg body wt. and maintained by further intraperitoneal or intravenous injections of up to a maximum of 1 ml. once or twice during the experiment as required. A long median incision was made in the extended neck of the dog and the thyroid glands were located. The heated thermocouple was inserted into the right thyroid lobe through a needle in which a shallow gutter had been previously made; the needle was withdrawn, leaving the flexible recorder *in situ*. The recorder was then tied in position, care being taken not to occlude the superior thyroid artery. The 'cold junction', which served as the reference point, was mounted in a polyethylene tube of small cross-sectional area. This was introduced into the right brachial artery and passed along it until the 'cold junction' came to lie in the aortic arch. An intravenous cannula was tied into the left femoral vein and blood pressure was recorded in the right femoral artery through a cannula connected to a mercury manometer and to a Wigger's type optical capsule mounted inside the dark chamber of the recording apparatus. The thermocouples were connected to the recording apparatus and the experiment started.

## RESULTS

*Resting blood flow.* It was observed in these experiments that spontaneous variations in resting $\delta k$ values occurred which in some experiments were small but in others large. For example, in one experiment the spontaneous $\delta k$ variation was from $5{\cdot}1 \times 10^{-4}$ to $0{\cdot}71 \times 10^{-4}$; in another experiment, the range of spontaneous change was from $4{\cdot}3 \times 10^{-4}$ to $3{\cdot}6 \times 10^{-4}$. Such spontaneous variation in resting $\delta k$ values made it difficult to assess the importance of small changes in blood flow in these experiments, therefore mean $\delta k$ values have been used to indicate the typical blood-flow change accompanying each type of experiment.

*Glucose.* The effect of glucose on thyroid blood flow and metabolic heat production was determined in ten experiments. Glucose was given through the intravenous cannula in the dose of 2g/kg body wt., being dissolved in 40 ml. normal saline and slowly injected by means of an infusion machine which delivered at the rate of 0·5 ml./min. The changes in $\delta k$ values and calculated excess temperature are shown in Table 1 and a typical response is shown in Fig. 1.

It can be seen from Table 1 that the change in $\delta k$ values started within 10 min of glucose injection; in eight experiments there was a considerable initial rise in $\delta k$ values ranging from 50 to 60% increase over the resting value. Only in two experiments was there an initial fall, of the order of

1·7 and 5·6 % of resting $\delta k$ values. This was followed in all experiments by a rise to a maximum value in 40 min after the injection of glucose. The $\delta k$ value then began to fall again, 45 min after glucose administration. A typical result is shown in Fig. 1*A*.

In nine experiments there was a rise in the calculated excess temperature which varied in extent from 4·5 to $47{\cdot}0 \times 10^{-2}$ °C. In only one experiment was there a fall of $21{\cdot}6 \times 10^{-2}$ °C (Table 1). The effect of glucose on excess

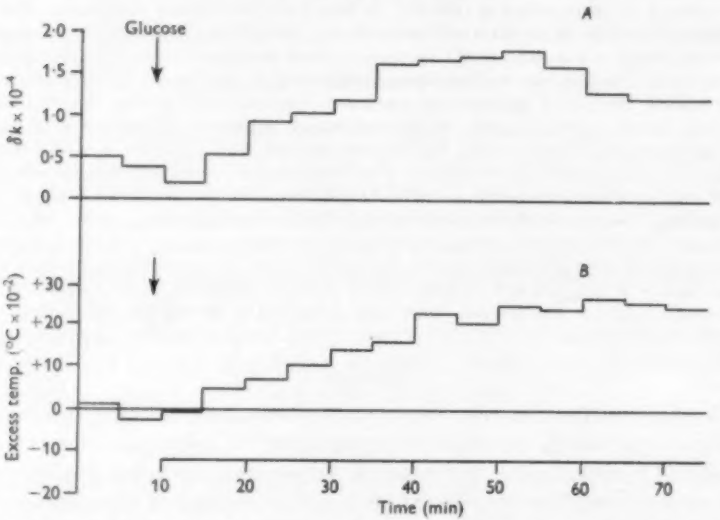



Fig. 1. Typical effect of glucose (I.V. infusion): *A*, on blood flow ($\delta k$) and *B*, on metabolic heat production (excess temperature) in a dog's thyroid gland.

TABLE 1. The effect of glucose on thyroid blood flow and calculated excess temperature. Mean rise in blood glucose = 55 % ± 2·10

| Expt. no. | Blood flow: Average initial $\delta k$ | Blood flow: Average $\delta k$ 10 min after glucose | Blood flow: Average $\delta k$ 60 min after glucose | Maximum change in excess temperature (°C × $10^{-2}$) | Time to maximum effect (min) |
|---|---|---|---|---|---|
| 1 | 4·45 | 7·75 | 2·68 | 4·5 | 40 |
| 2 | 0·31 | 1·36 | 1·39 | 28·0 | 45 |
| 3 | 9·0 | 13·40 | 2·10 | 42·0 | 50 |
| 4 | 1·5 | 3·0 | 0·38 | 20·2 | 38 |
| 5 | 1·2 | 3·5 | 1·05 | 17·0 | 36 |
| 6 | 3·0 | 22·8 | 0·36 | 47·0 | 45 |
| 7 | 5·35 | 5·05 | 3·2 | −21·6 | 40 |
| 8 | 0·41 | 0·95 | 1·1 | 26·5 | 50 |
| 9 | 1·88 | 2·37 | 2·9 | 7·0 | 56 |
| 10 | 3·42 | 3·36 | 1·37 | 33·1 | 45 |

temperature sometimes started quickly, but always within 25 min of the injection of glucose, and peak values were reached 60 min after the start of the experiment. In all these experiments the quantity of glucose given (2g/kg body wt.) increased the blood glucose level by a mean value of 55% ± 2·10 (S.E. of mean).

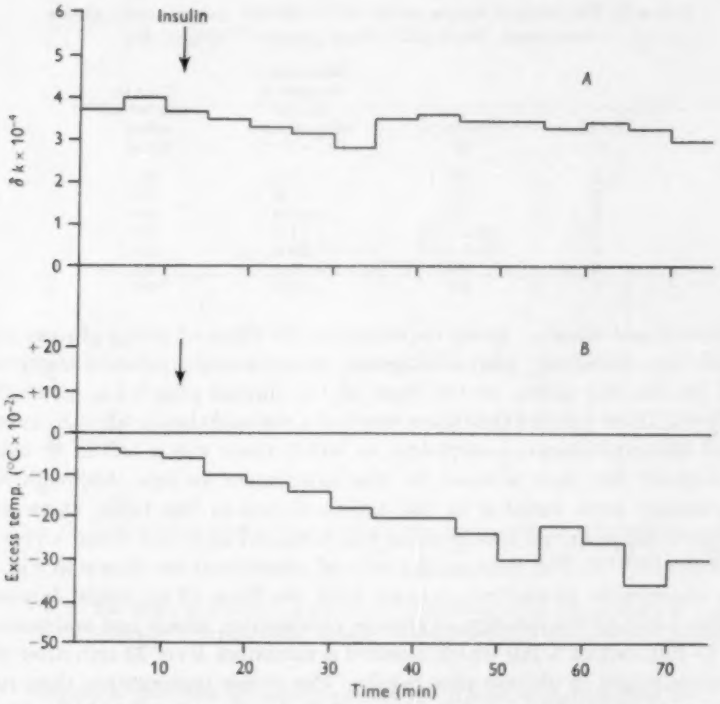



**Fig. 2.** Effect of insulin (I.V. injection): *A*, on blood flow ($\delta k$) and *B*, on metabolic heat production (excess temperature) in a dog's thyroid gland.

*Insulin*. The effect of insulin on thyroid blood flow and metabolic heat production was determined in seven experiments. 3 i.u./kg body wt. of insulin (insulin A.B., British Drug Houses, Ltd., 40 i.u./ml.) was given intravenously. The changes in $\delta k$ values and the calculated excess temperatures are given in Table 2 and the pattern of a typical response is shown in Fig. 2.

In five experiments there was no change in $\delta k$ values, but in two experiments there was a fall of 25 and 35% of the initial mean $\delta k$ values respectively. In all seven experiments insulin caused a fall in the calcu-

lated excess temperatures (Table 2) which varied in extent from $-7{\cdot}1$ to $-42 \times 10^{-2}$° C. The fall in excess temperature occurred within 5 min of the injection of insulin, the temperature remaining depressed in all the experiments for 60 min. Figure 2 shows a typical experimental result. There was a mean fall of $50\% \pm 1{\cdot}30$ in blood glucose in these experiments.

TABLE 2. The effect of insulin on thyroid blood flow and calculated excess temperature. Mean fall in blood glucose = $50\% \pm 1{\cdot}30$

| Expt. no. | Change in $\delta k$ | Maximum changes in excess temperature (°C $\times 10^{-2}$) | Time to maximum effect (min) |
|---|---|---|---|
| 1 | Nil | − 7·1 | 45 |
| 2 | Nil | −11·2 | 50 |
| 3 | Nil | −42·0 | 48 |
| 4 | 19·2–12·5 | −17·0 | 60 |
| 5 | 18·3–13·3 | −29·0 | 48 |
| 6 | Nil | −15·2 | 60 |
| 7 | Nil | −16·2 | 60 |

*Glucose and insulin.* In six experiments the effect of giving glucose and insulin simultaneously was investigated. An intravenous infusion of glucose and insulin was given, in the dose of 1 g glucose plus 1 i.u. insulin/kg body wt. Table 3 shows that there was first a rise and then a fall in $\delta k$ values in all the experiments except one, in which there was a fall in $\delta k$ value throughout the time allowed for the experiment to run. Although the $\delta k$ changes were variable to the extent shown in the table, there was always a fall in excess temperature which ranged in extent from $-12{\cdot}9$ to $-56{\cdot}0 \times 10^{-2}$° C. The changes in a typical experiment are shown in Fig. 3. The changes in excess temperature took the form of an initial increase within 5 min of the infusion of glucose plus insulin, which was maintained for 15 min before a fall which reached a minimum level 35 min after the administration of glucose plus insulin. The excess temperature then rose to normal within 15 min of maximum fall. On the other hand, the blood flow rose within 10 min of the injection of glucose plus insulin and fell to a minimum level within 60 min. In five experiments there was no difference between the resting blood glucose values and those after the injection of glucose and insulin, whereas in one experiment there was a rise of $30{\cdot}3\% \pm 2{\cdot}45$.

*Adrenaline.* In eight experiments adrenaline was infused intravenously in the dose of 8 $\mu$g/min, in order to investigate the effect of adrenaline on the rate of blood flow and heat production of the thyroid gland. In four of these experiments there was an increase in mean excess temperature ranging in extent from 0·05 to $31{\cdot}3 \times 10^{-2}$° C and in the others a fall in mean excess temperature ranging from $-2{\cdot}9$ to $-38{\cdot}0 \times 10^{-2}$° C. These

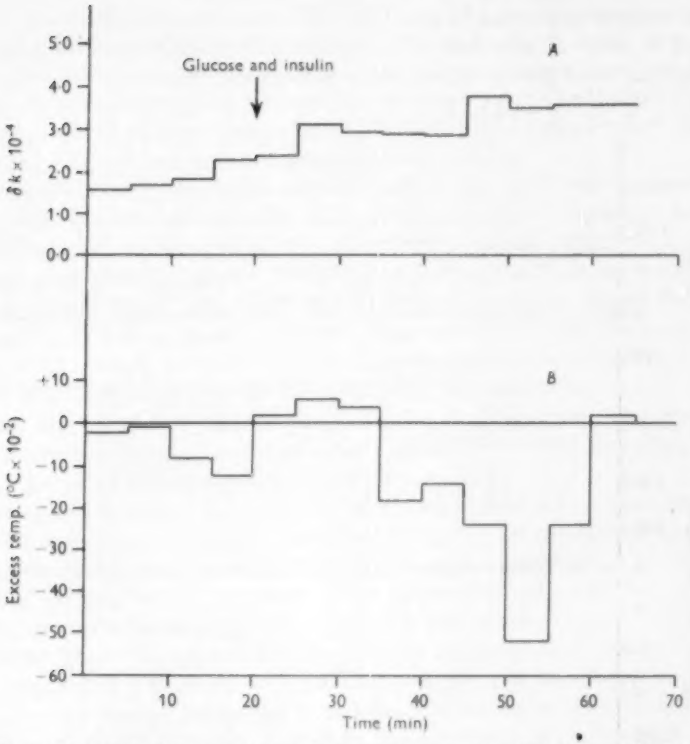



**Fig. 3.** The effect of glucose and insulin (I.V. infusion): *A*, on blood flow ($\delta k$) and *B*, on metabolic heat production (excess temperature) in a dog's thyroid gland.

**TABLE 3.** The effect of insulin combined with glucose on thyroid blood flow and calculated excess temperature

| Expt. no. | Blood flow: Average initial $\delta k$ | Blood flow: Average $\delta k$ 10 min after glucose insulin | Blood flow: Average $\delta k$ 60 min after glucose insulin | Maximum change in excess temperature (°C × $10^{-2}$) | Time to maximum effect (min) | Average blood glucose (% of resting level) |
|---|---|---|---|---|---|---|
| 1 | 4·00 | 7·90 | 1·48 | −28·8 | 40 | No change |
| 2 | 1·57 | 7·00 | 0·31 | −12·9 | 45 | No change |
| 3 | 5·35 | 3·51 | 3·96 | −33·0 | 60 | 30·3 ± 2·45 rise |
| 4 | 0·41 | 1·20 | 1·60 | −56·0 | 40 | No change |
| 5 | 1·88 | 3·08 | 3·52 | −40·5 | 48 | No change |
| 6 | 3·42 | 1·71 | 1·90 | −49·2 | 52 | No change |

results are shown in Table 4 and a typical result is shown in Fig. 4. In all these experiments it can be seen that there was a constant fall in $\delta k$ value, which in some experiments is of considerable magnitude. There was no change in blood glucose level in any of these experiments.

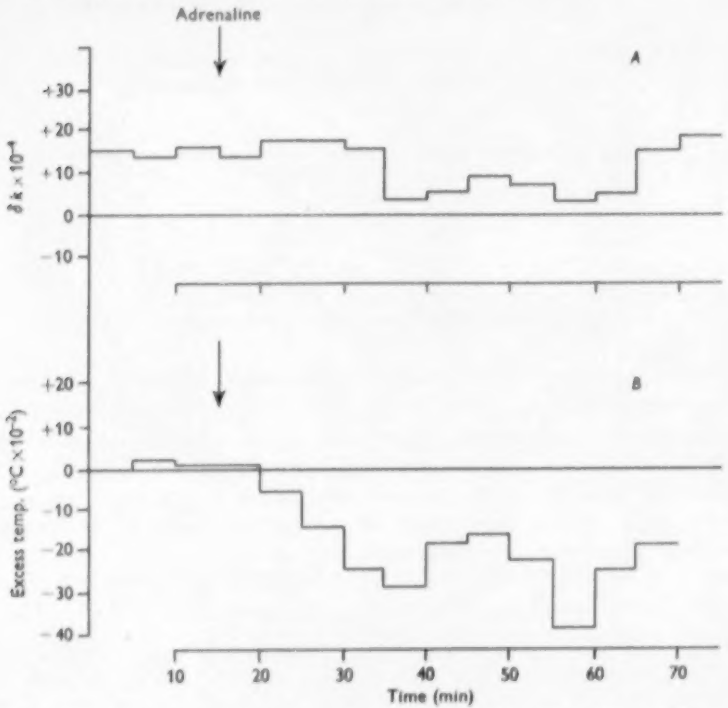



*Fig.* 4. The effect of adrenaline (I.V. infusion): *A*, on blood flow ($\delta k$) and *B*, on metabolic heat production (excess temperature) in a dog's thyroid gland.

TABLE 4. The effect of adrenaline on thyroid blood flow and calculated excess temperature. No change in resting blood glucose level

| Expt. no. | Change in $\delta k$ from | to | Change in mean excess temperature (°C × $10^{-2}$) | Time to maximum effect (min) |
|---|---|---|---|---|
| 1 | 4·45 | 3·45 | +5·7 | 40 |
| 2 | 0·31 | 0·15 | −9·1 | 45 |
| 3 | 9·00 | 2·11 | −2·9 | 50 |
| 4 | 1·50 | 0·25 | −12·6 | 48 |
| 5 | 1·80 | 0·4 | −38·0 | 60 |
| 6 | 3·00 | 0·4 | +31·3 | 50 |
| 7 | 4·00 | 0·9 | +8·0 | 40 |
| 8 | 1·60 | 0·3 | +0·05 | 45 |

DISCUSSION

The application of internal calorimetry to the study of blood flow and heat production in internal organs has been fully validated (Dosekun *et al.* 1960). In the present work the methods were applied to the thyroid gland mainly with the object of investigating its blood flow and metabolic heat production in relation to its carbohydrate metabolism.

The effect of maintaining a raised blood glucose level was to produce initial marked increments in blood flow, accompanied by marked increments in metabolic heat production. The blood flow then fell within 45 min of beginning glucose infusion. The present work gives little indication as to the mechanism behind these effects. It is tempting to regard the increased blood flow as secondary to the metabolic effect. Against this, however, is the observation that when glucose and insulin were combined and blood glucose changes reduced to a minimum, metabolic heat production fell but the blood flow changed in much the same way and to a similar extent as when glucose was administered alone. It is therefore difficult to relate the blood-flow changes to blood glucose levels.

The elevation of heat production with glucose is also difficult to account for and the present work gives no clear indication of the metabolic processes which might be involved. In both liver and muscle raising the levels of blood glucose had the opposite effect, metabolic heat production being inhibited in the presence of high levels of blood glucose.

Insulin alone had no measurable effect on thyroid blood flow even in the comparatively large doses in which it was given in these experiments; but in every case it produced a marked depression of metabolic heat production, which occurred soon after the injection of insulin and continued till the end of the experiments. This lowering of heat production might be regarded as either secondary to the hypoglycaemia produced by insulin or as a direct action of insulin within the glandular cells. However, in the experiments where insulin infusion was combined with glucose and the blood glucose level was thus prevented from changing, the result was still a depression of metabolic heat production. It must be concluded, therefore, that insulin has a direct and rapid depressive action on metabolic heat production within the thyroid gland. In other tissues such as liver, muscle and brain, the initial effect of insulin, even in the presence of low blood glucose levels, was to enhance heat production and only when hypoglycaemia was very prolonged was this effect superseded by a depression.

It might be considered that these effects are related to adrenaline release. In the present experiments, however, adrenaline had no consistent effect on metabolic heat production. It consistently lowered blood flow, and

since blood pressure rose it must be regarded as vasoconstrictor to the thyroid blood vessels.

It is clear that, in regard to insulin and carbohydrate metabolism, striking differences exist between the thyroid gland and the other tissues referred to above. It might be relevant to suggest that in liver and muscle glycogen break-down has been related to heat production effects (Dosekun *et al.* 1960). There is strong evidence to suggest even in the brain the existence of some substrate usable for the local formation of glucose. It is likely, however, that in the thyroid gland there is no such readily available substrate, either in the form of stored glycogen or any other form, and that the present findings are explicable on the basis of a direct dependence of the gland on blood glucose levels. On the present evidence, however, this cannot be more than speculation and a complete elucidation of the problem must await further investigation.

## SUMMARY

1. The technique of internal calorimetry has been used to investigate the metabolic heat production and blood flow of the thyroid gland.

2. Insulin has been found to depress the metabolic heat production of the thyroid gland without any effect on its blood flow.

3. Glucose has been found to increase both the heat production and blood flow of the thyroid gland.

4. The effect of adrenaline on blood flow changes has been shown to be that of vasoconstriction but its effect on metabolic heat production is inconsistent; sometimes a rise, at other times a fall being caused by adrenaline.

5. The probable influence of insulin and glucose on the carbohydrate metabolism of the thyroid gland has been discussed.

6. A comparison has been made between the effects of glucose and insulin on the thyroid, liver, muscle and brain and it is clear that in regard to heat production, these substances produce different effects in the thyroid gland, liver and muscle. As regards blood flow changes, the effect of glucose on the three tissues is similar whereas they differ with insulin.

## REFERENCES

Carlyle, A. & Grayson, J. (1956). Factors involved in the control of cerebral blood flow. *J. Physiol.* **133**, 10–30.

Dosekun, F. O., Grayson, J. & Mendel, D. (1960). The measurement of metabolic and vascular responses in liver and muscle with observations on their responses to insulin and glucose. *J. Physiol.* **150**, 581–606.

Grayson, J. (1952). Internal calorimetry in the determination of thermal conductivity and blood flow. *J. Physiol.* **118**, 54–72.

Grayson, J. & Kinnear, T. (1958). Vascular and metabolic responses of the liver to insulin. *J. Physiol.* **144**, 52–7.

Grayson, J. & Mendel, D. (1957). Observations on the intrahepatic flow interactions of the hepatic artery and portal vein. *J. Physiol.* **139**, 167–177.

*J. Physiol.* (1961), **157**, *pp.* 513–528
*With 2 plates and 4 text-figures*
*Printed in Great Britain*



# THE MORPHOLOGY OF INTRAFUSAL MUSCLE FIBRES IN THE CAT

BY D. BARKER AND JANKI L. GIDUMAL
*From the Department of Zoology, University of Hong Kong*

(*Received* 1 *February* 1961)

It has long been recognized that the muscle spindle is composed of muscle fibres of large and small diameter (see e.g. Sherrington, 1894; Cuajunco, 1927, 1940), but the claim that the fibres branch in their course through the spindle has been a matter of controversy. Branching was held to occur by the majority of workers from Weismann (1861) to Denny-Brown (1928), though it is probable that many of the earlier claims were prejudiced by a belief that spindles served as centres of muscle growth. According to Sherrington (1894) and Ruffini (1898), large 'parent' fibres in the proximal pole of the spindle split longitudinally to form smaller 'daughter' fibres, which course through the equatorial region to taper off as long tendinous filaments in the distal pole. However, Batten (1897) and recently Häggqvist (1960) maintain that branching in one pole is followed by reuniting of the fibres in the other. Forster (1894) holds that the branching and reuniting of fibres occurs throughout the length of the spindle, and Cilimbaris (1910) adds that they also anastomose so as to form a syncitial network in the equatorial region reminiscent of cardiac muscle. Against this it has been urged (Baum, 1900; Cuajunco, 1927, 1940; Barker, 1948; Gray, 1957; Boyd, 1958; Cooper, 1960) that intrafusal fibres remain single, and that the tapering of short fibres alongside longer ones may have been mistaken for instances of branching. At a preliminary stage of the present study we became convinced that branching does occur, and it appeared to us that Forster's observations were correct (Barker, 1959). Further investigation, however, led us to the conclusion that branching is mainly exhibited by the small intrafusal fibres (Barker & Gidumal, 1960), and Boyd (1960), reversing his earlier opinion (1958), arrived at a similar conclusion. The present paper deals with this matter in detail.

## METHODS

We have made the majority of observations on spindles from the rectus femoris muscle of the cat, but have also examined spindles from the teres minor, sternohyoid, 7th internal intercostal, tenuissimus, and vastus lateralis muscles of this animal. The bulk of the

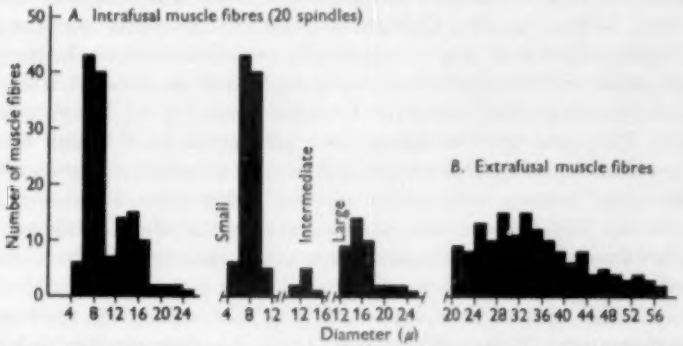
material was embedded in paraffin, cut serially and transversely at 12 $\mu$, and stained with haematoxylin and eosin. The muscles were fixed *in situ* in either Bouin's or Zenker's fluid, and were subsequently dissected out in either 70% alcohol or water according to the fixative employed. Other material studied included longitudinal paraffin sections; transverse celloidin, 'Nonex', and frozen sections; teased gold-chloride preparations (Gairns's method); and macerated preparations in which either nitric acid, gastric juice, or collagenase was used in an endeavour to dissociate the intrafusal muscle fibres. In the application of these various methods observations were made on 246 spindles, of which twenty were of the tandem type, but of this total the most detailed information was obtained from the graphic reconstruction of thirty-two spindles (including three tandems) cut in serial transverse section. In the first five reconstructions, camera lucida drawings of the sections were made at magnifications of $\times 430$ or $\times 600$. Initially every section in a series was drawn, but this was later reduced to every fifth 12 $\mu$ section when it became evident that the complete process of fibre branching occupied a length of 60–90 $\mu$. When branching was detected, however, every section in that part of the series was drawn. The drawings were then converted into a projection-plan of the spindle, at the same magnification, in which the intrafusal bundle is presented as if divided sagittally and the fibres in each half arranged in a horizontal plane. In reducing these projection-plans to more manageable dimensions, the length of the fibres was accurately represented but their width exaggerated. In reconstructions of twenty-seven further spindles sketches of every fifth section were substituted for camera lucida drawings, and projection-plans made with the aid of these and measurements of the lengths and diameters of the individual fibres. The diameters of fibres that were not circular in section were recorded as the average of their maximum and minimum widths. In a few selected instances where the bifurcation of a fibre could be demonstrated to the best advantage, serial photographs were taken using an oil-immersion objective.

## RESULTS

The intrafusal bundle in single spindles from rectus femoris consists of a group of four to twelve muscle fibres in which those of small diameter and short length usually predominate over those which are larger and longer. Constant features of the bundle are its encapsulation, suspension in a lymphatic space, and changes in nucleation which occur in the equatorial region associated with the sensory innervation. The location of this region appears to be related to the position of the spindle in the muscle, and the polar regions on either side of it are usually of unequal length (Barker & Ip, 1961). There is no constant feature which is characteristic of one pole and not the other, but it is convenient to designate them as proximal and distal with respect to their orientation with the origin and insertion of the extrafusal muscle bundles. We regard a polar region of a muscle spindle as extending from the juxta-equatorial region to the furthest extremity of the origin or insertion of the muscle fibres composing it. However, the term 'spindle' is sometimes restricted by others to include only that part of the muscle bundle invested by the capsule, the polar extremities being regarded as coincident with the capsular extremities. Thus Häggqvist (1960) describes muscle fibres as entering or emerging from the spindle at one or other pole, and distinguishes between their extra- and intrafusal course. This can only lead

to confusion in view of the wide use of the term 'spindle' to denote the whole proprioceptor. Tandem spindles may be regarded as consisting of a number of single spindle units connected together by a few intrafusal fibres of large diameter which run a continuous course through the successive encapsulations.

The two main types of intrafusal muscle fibre present in all spindles are those which are large and long and possess the characteristic nuclear bag and myotube regions described by Barker (1948), and those which are small and short with a single central row of nuclei in the equatorial region (the nuclear-chain fibres of Boyd, 1960). Both length and diameter measurements of the fibres composing the intrafusal bundle are therefore bimodal. However, in some spindles there are fibres present whose




Text-fig. 1. *A*. Mid-polar diameter histograms of a sample of 142 muscle fibres belonging to 18 single and 2 tandem spindles from the rectus femoris muscle of a 2 kg cat. The histogram on the left is broken down on the right in terms of the 94 small, 8 intermediate, and 40 large fibres in the sample. *B*. Diameter histogram of a random sample of 150 extrafusal muscle fibres from the same muscle. Measurements made on Zenker-fixed transverse paraffin sections.

characteristics make it necessary to recognize a third type which we shall designate as intermediate. In a sample of eighteen single and two tandem spindles from the rectus femoris muscle of a 2 kg cat (C 50), there were ninety-four small, forty large, and eight intermediate fibres whose mid-polar diameters were 4–12$\mu$ (mean 7·5$\mu$), 12–26$\mu$ (mean 16·9 $\mu$), and 10–16$\mu$ (mean 12·6$\mu$) respectively (see Text-fig. 1). The diameter range of a random sample of 150 extrafusal muscle fibres from the same muscle was 20–58$\mu$ (mean 35$\mu$). The length of intrafusal fibres is such that the small fibres may be about half as long as the large ones. In ten single rectus femoris spindles 7·5–8·5 mm long, the length range of thirty-five small fibres was 4·0–6·1 mm, of seventeen large fibres 7·0–8·5 mm, and of five intermediate fibres 6·4–7·8 mm.

From one to three, usually two, large fibres are present in all spindles. They tend to retain the same position in the intrafusal bundle, and do not branch. Their diameter undergoes little change except at the extreme ends, where tapering occurs, and in the myotube regions, where there is typically a decrease of two or three microns. The small fibres also narrow down in the equatorial region, their diameter becoming little more than that of each vesicular nucleus in the nuclear chain. About one in five bifurcates, and the two fibres produced often reunite. If parent fibres only are counted, the number of small fibres present in a spindle is from one to eight, and is usually four or five. Their position in the intrafusal bundle undergoes frequent changes as they twist among themselves and cross over the large fibres. The intermediate type of fibre resembles the large in that it possesses nuclear-bag and myotube regions, but the bags tend to have smaller aggregations of vesicular nuclei, and the myotube regions show a more pronounced decrease in diameter. In the polar regions their variation in diameter is such that in isolated transverse sections they may easily be taken for small fibres (cf. Pl. 1, figs. 2 and 5, fibre *a*). They also tend to change their position in their course through the intrafusal bundle. About one out of three spindles possesses fibres of this type; usually only one is present, never more than two. They occasionally bifurcate; in one exceptional case the fibres produced were observed to reunite. While these fibres are of medium size in the intrafusal fibre diameter spectrum, a medium-sized fibre is not necessarily of the intermediate type; the equation of fibre size with fibre type in our preliminary note (Barker & Gidumal, 1960) was thus an over-simplification.

In the process of bifurcation, as seen in optimum transverse sections (see Pl. 1; Pl. 2, figs. 1–9), a small or intermediate type fibre loses its circular contour and increases in size to assume an oval shape. This becomes constricted into the form of a figure of eight before finally splitting into two. The complete process occupies a distance of 60–90$\mu$. In the reuniting of small fibres the reverse sequence occurs. The two fibres produced by branching are usually unequal in size but their combined diameters are initially equal to that of the parent fibre. After a short distance, however, both fibres increase in diameter, the larger of the two regaining the diameter of the parent fibre. For example, a small fibre 8$\mu$ in diameter will typically branch into two fibres which initially measure 5 and 3$\mu$ in diameter, and subsequently increase to diameters of 8 and 5$\mu$. The identity of the parent fibre thus tends to be retained after division. This is particularly marked in the case of the intermediate type fibres which branch, for when the fibres produced pass through the equatorial region the larger of the two has a nuclear bag and myotube region while

the other has a nuclear chain (see Text-fig. 2; Pl. 2, figs. 1–9). Bifurcation, with the branches running towards the equator, occurs in the proximal pole or in the proximal part of the equatorial region; in tandem spindles it occurs in the equivalent parts of the single spindle units that are linked together. When small fibres branch, the fibres produced may either continue through the intrafusal bundle and taper off, or reunite in the distal pole or distal part of the equatorial region. We have never observed a small fibre to give a distally directed branch in the distal pole, but in one instance an intermediate fibre gave rise in the distal pole to a short branch running away from the equator (see Text-fig. 4; and Pl. 1). The branching of intermediate fibres is easier to detect and to demonstrate owing to their larger size. It should be possible to obtain convincing evidence of branching from teased and dissociated material, but our attempts to do so have so far proved unsuccessful.

The process of branching or reuniting is quite distinct from the condition where one fibre tapers off alongside another (see Pl. 2, figs. 10–13). Tapering takes place over a distance of several hundred microns, the shortest length being about three times that occupied by branching or reuniting. A fibre tapers off usually over a distance of 300–500$\mu$, but distances of up to 800$\mu$ may occur. The shortest tapering lengths tend to occur within the polar regions at the ends of the small fibres, while at the extremities of the spindle the larger fibres taper off as longer tendinous filaments. In transverse section tapering is marked by a progressive loss in diameter, and ultimately by a loss of circular contour and myofibril content. The final tendinous wisp of a small fibre usually merges with the endomysium of a larger fibre or the axial sheath, and the ends of the larger fibres merge with either tendon, aponeurosis, perimysium, or the endomysium of neighbouring extrafusal fibres.

In transverse section some of the muscle fibres composing the intrafusal bundle may exhibit Krüger's (1952) 'Fibrillenstruktur', others a 'Felderstruktur' (fibrillar and areal patterns of Gray, 1958). However, it is not possible to relate structural pattern categorically to fibre type, since the pattern does not remain the same throughout the length of each fibre. For most of their length all fibres are fibrillar, and the fibrillar pattern is evident in regions where branching or reuniting occurs. The areal pattern may occur sporadically in the polar regions, but it is characteristic of the juxta-equatorial regions. When there is a change from the fibrillar to the areal pattern it usually occurs in the majority of the fibres in the intrafusal bundle. Each fibre is usually areal for part of its length, the total distance amounting to several hundred microns, seldom more than 1 mm. In fibres of the large and intermediate types this forms only a small part of their total length so that their structural pattern is predomi-

nantly fibrillar. In small fibres the ratio of areal length to fibrillar length is higher, owing to their shorter total length, and by comparison with the large fibres they give the impression of being predominantly areal. However, the areal portions of small fibres seldom exceed 35% of their total length, and the percentage is often considerably less. Boyd (1960) implies that large fibres are exclusively fibrillar and small fibres exclusively areal, but we have never observed this condition. It may be significant that the areal pattern appears to be most prevalent in the juxta-equatorial regions of the spindle where the motor innervation tends to be most profuse. If fibrillar and areal patterns are indicative of fast and slow

TABLE 1. Number and nature of muscle fibres belonging to eighteen spindles from cat rectus femoris

*s*, single fibres; *bt*, fibres bifurcating and tapering; *br*, fibres bifurcating and reuniting

| | Large type | Intermediate type | | | Small type | | | total | |
|---|---|---|---|---|---|---|---|---|---|
| Cat | *s* | *s* | *bt* | *br* | *s* | *bt* | *br* | min. | max. |
| C8 | 2 | 1 | — | — | — | — | 1 | 4 | 5 |
| C24 | 2 | 1 | — | — | 3 | 1 | 1 | 8 | 10 |
| C60 | 2 | 1 | — | — | 4 | — | 1 | 8 | 9 |
| C50 | 3 | — | — | — | 3 | 1 | — | 7 | 8 |
| C50 | 2 | 1 | — | — | 3 | 1 | — | 7 | 8 |
| C50 | 2 | — | — | — | 5 | — | — | 7 | 7 |
| C50 | 2 | 1 | — | — | 4 | — | 1 | 8 | 9 |
| C50 | 1 | 1 | — | — | 5 | — | — | 7 | 7 |
| C50 | 1 | — | — | — | 6 | — | 1 | 8 | 9 |
| C50 | 2 | — | — | — | 4 | — | — | 6 | 6 |
| C50 | 1 | — | — | — | 5 | — | — | 6 | 6 |
| C50 | 2 | 1 | — | — | 5 | — | — | 8 | 8 |
| C50 | 3 | — | — | — | 3 | 1 | 1 | 8 | 10 |
| C50 | 1 | — | — | — | 3 | 1 | — | 5 | 6 |
| C50 | 2 | 1 | — | — | 3 | — | — | 6 | 6 |
| C50 | 2 | — | — | — | 3 | — | 1 | 6 | 7 |
| C50 | *2 | 1 | 1 | — | 5 | 3 | — | 12 | 16 |
| C63 | 1 | — | 1 | (1) | 7 | — | — | 9 | 10 (+1) |
| Totals | 33 | 9 | 2 | (1) | 71 | 8 | 7 | 130 | 147 (+1) |
| | | 11 (+1) | | | 86 | | | | |

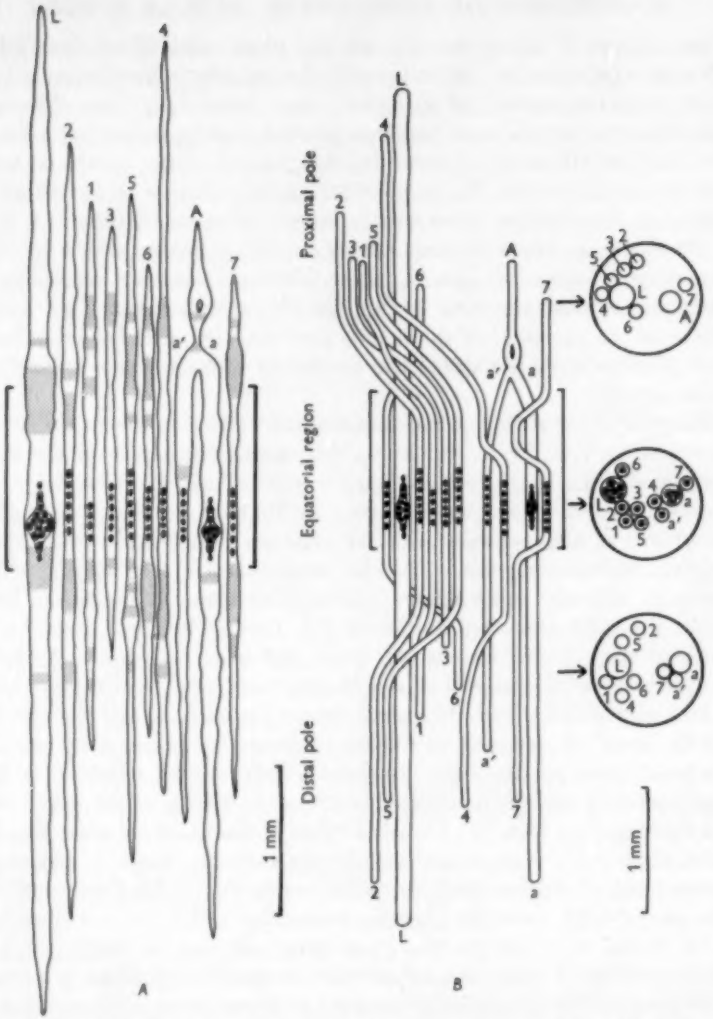
* Tandem spindle.

contraction, as Krüger (1952) maintains, the quickness or slowness of intrafusal muscle fibres must be a matter of degree, for in the individual fibre the two patterns are not mutually exclusive. In this connexion it may be noted that Krüger's observations on the structural pattern of extrafusal muscle fibres were made on individual transverse sections, no fibres being traced throughout their length; the same is true of Gray's (1958) study of frog muscle.

The number and nature of the muscle fibres belonging to eighteen spindles traced in transverse sections of the rectus femoris muscles of four adult cats are shown in Table 1. There are eighty-six small fibres

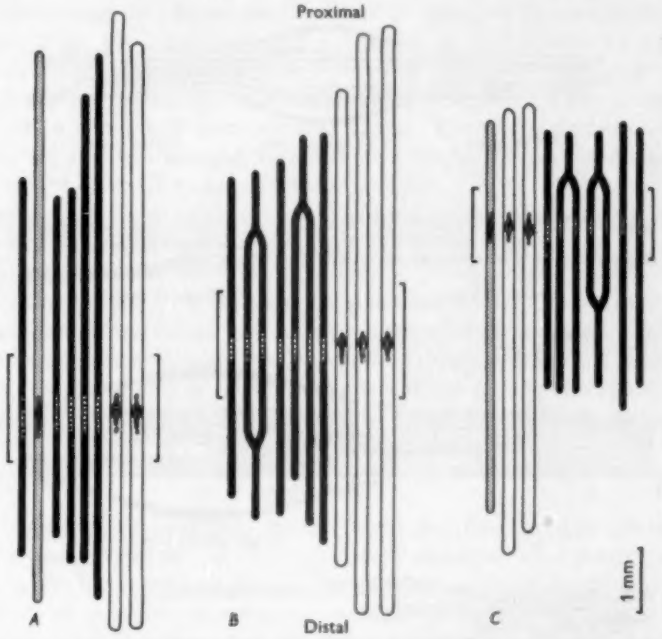
in the sample, of which seventy-one are single, and fifteen, or 17·4%, bifurcate. Intermediate fibres occur in ten spindles, there being a total of eleven in the sample, of which two show branching. One of these is exceptional in that the fibre branches twice, reuniting almost immediately after the first bifurcation before branching again; this sequence is shown as *br* (1) and *bt* 1 in the Table (see C63). In six spindles all the fibres are single, an intermediate fibre being present in three of them. A total of 130 single and parent fibres belong to the seventeen single spindles, an average of seven per spindle; if the fibres produced by branching are taken into account the total is 147, and the average eight. The tandem spindle in the sample is of the double kind and accounts for a maximum total of sixteen fibres, double the maximum average fibre content of a single spindle.

Many of the features of intrafusal muscle fibres so far described are illustrated in Text-fig. 2, which is a diagrammatic analysis of the intrafusal bundle of a single rectus femoris spindle consisting of one large, one intermediate, and seven small fibres. In Text-fig. 2*A* the muscle fibres are arranged schematically side by side and enclosed within square brackets representing the equatorial encapsulation. The length of this region, in this and other figures, corresponds with the equatorial limits of the periaxial space (cf. Barker & Ip, 1961). The scale used for the width of the muscle fibres is ten times that used for length. Variation in muscle fibre diameter is shown on the basis of measurements taken at intervals of every fifth 12$\mu$ serial transverse section, and the distribution of areal, as opposed to fibrillar, pattern is indicated by shading. The small fibres remain single throughout their lengths, which range from approximately two-fifths to three-quarters the length of the large fibre. The intermediate fibre (*A*) branches twice in the proximal pole. The first bifurcation is the exceptional one already noted in Table 1, and results in two fibres of similar diameter, which rejoin after a brief course of 72$\mu$. The second bifurcation occurs 360$\mu$ further on and is the one illustrated in Pl. 2, figs. 1–9. Of the two fibres produced, one (*a*) displays typical characteristics of an intermediate fibre in that its diameter is variable and the equatorial nucleation consists of myotube regions and a small nuclear bag, while the other (*a'*) has the characteristics of a small fibre in both diameter and equatorial nucleation (see Pl. 2, figs. 6–8). The distribution of fibrillar and areal structure in the fibres illustrates the typical prevalence of the areal pattern that occurs in the juxta-equatorial regions. In this spindle the nuclear bags and chains lie towards the distal end of the equatorial region, as is often the case. In Text-fig. 2*B* the spindle is shown in schematic projection-plan in order to illustrate the change of position of fibres in their course through the intrafusal bundle;




Text-fig. 2. Diagrammatic analysis of a single rectus femoris cat spindle comprising one large fibre (L), one intermediate fibre (A) bifurcating into *a* and *a'*, and seven small fibres (1–7). *A*. Each fibre's length and level of origin and insertion is accurately shown, but scale used for width is $\times 10$ that used for length. Diameter variation is shown on basis of $60\mu$ interval measurements; stippling indicates areal, as opposed to fibrillar, pattern. *B*. Projection-plan and three schematic transverse sections illustrating changes of fibre position; scale applies to plan only, scale for fibre width being $\times 8$ that for length. Fibre diameters standardized as $17\mu$ large, $13\mu$ intermediate, $8\mu$ small. In both *A* and *B* square brackets indicate limits of equatorial encapsulation; nuclei schematic, but length of each nucleated region accurate. Compare with Pl. 2, figs. 1–9.

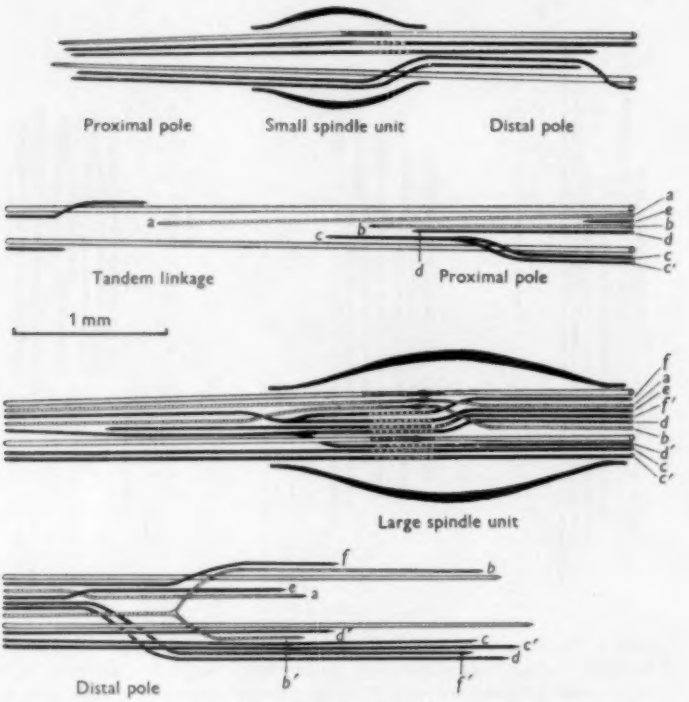
the appearance in transverse section is shown diagrammatically at three key levels alongside. The sinuous course of the small fibres, and the stable course of the large, are typical.




Text-fig. 3. Schematic diagrams of the fibres composing three single rectus femoris cat spindles. Large fibres white, intermediate fibres stippled, small fibres black. Fibre diameters standardized as in Text-fig. 2*B*, and shown on a scale ×10 that used for length. Square brackets indicate limits of equatorial encapsulation; nuclei schematic but length of each nucleated region accurate.

As a further illustration of the composition of the intrafusal bundle of single spindles, the muscle fibres of three spindles from rectus femoris are drawn in stylized form, side by side, in Text-fig. 3 *A–C*. An intermediate fibre is present in spindles *A* and *C*, but not in *B*. In *A* all the fibres are single, while in both *B* and *C* a small fibre bifurcates, and another bifurcates and reunites. Spindles *B* and *C* provide good examples of how the composition of the intrafusal bundle fluctuates at different levels owing to the different lengths of the fibres composing it and the occurrence of branching and reuniting. The maximum number of fibres is always present at mid-equatorial level.

Finally, in Text-fig. 4 a tandem spindle is illustrated in schematic projection-plan, the equatorial encapsulations being indicated as diagrammatic fusiform swellings. The tandem is of the typical double type described by Barker & Ip (1961), consisting of a small capsule enclosing




Text-fig. 4. Projection-plan of double tandem spindle from cat rectus femoris shown as if cut into four portions. Large fibres white, intermediate fibres stippled, small fibres black. Scale applies to fibres only; scale for width $\times 2$ that for length, and diameters standardized. Equatorial encapsulations indicated as fusiform swellings; nuclei schematic, but length of each nucleated region accurate. Full description in text. Photographs of the bifurcation of fibre *b* are shown in Pl. 1.

a few muscle fibres linked, by two large fibres, to a large capsule in which the fibres are more numerous. In the small spindle unit four single small fibres accompany the two continuous large ones; the equatorial nucleation of the fibres is characteristically sparse and comprises three nuclear chains and three fibres in which polar nucleation is retained. In the large spindle unit, the large fibres are accompanied by two intermediate fibres (*a* and *b*) and four small (*c*–*f*). Fibre *a* remains single while *b*

branches in the distal pole to produce a short branch *b'*: it is this bifurcation that is illustrated in Pl. 1. Of the four small fibres, three branch (*c*, *d*, and *f*), while *e* remains single: none of the branching fibres reunite. Owing to differences of fibre length, and to branching, the numbers of fibres composing the large spindle unit, as counted at one-millimetre intervals from the proximal polar extremity to mid-equatorial region, increases in the progression 3, 7, 8, 9, 11. The equatorial nucleation comprises four nuclear bag and myotube regions belonging to the large and intermediate fibres, and seven nuclear chains. The stable position of the large fibres, and the changes in position of the small and intermediate fibres, are represented as accurately as possible.

It occasionally happens that a few extrafusal muscle fibres run a parallel course with a spindle and become enclosed within the capsule together with the intrafusal bundle. Baum (1900) and Cooper (1960) have made similar observations. On the rare occasions when this occurs the intrafusal and extrafusal fibres are always segregated in separate capsular compartments. In common with Häggqvist (1960), we have not observed supplementary bundles of intrafusal muscle fibres joining the spindle by penetrating the equatorial encapsulation as described by Cilimbaris (1910).

## DISCUSSION

The branching of intrafusal muscle fibres has long been a matter of dispute, partly because of the intrinsic difficulties of demonstrating it, and partly because of inadequate histological methods employed. For example, the practice of selecting sections at regular intervals in a transverse series and discarding the rest is a severe handicap to drawing any valid conclusion. A fibre branches within the space of a few transverse sections covering 60–90$\mu$, so that gaps in a series of e.g. 85$\mu$ (Swett & Eldred, 1960) are unlikely to reveal the process. Pronouncements made on the basis of studying serial longitudinal sections and teased gold-chloride preparations (e.g. Barker, 1948), or methylene blue preparations (e.g. Gray, 1957), are also of little significance for it is extremely difficult to trace individual members of the intrafusal bundle with certainty for any considerable length in such material. Dissociating methods also, in our experience, give equivocal results. The only adequate approach is to trace a fair sample of spindles from end to end in a complete series of transverse sections cut exactly at right-angles to the axis of muscle fibre orientation. Even in such material the small fibres, in particular, are often difficult to trace, and the chances of error are greater, for example, than in tracing nerve fibres of equivalent dimensions. The changes of diameter, position, and number of fibres in the intrafusal bundle, inter-

mixed with a profusion of nerve fibres and nuclei, often present a complex picture in which several interpretations are possible, and our present position has only been attained gradually, the stringency of criteria and rejection of ambiguities increasing together with sampling and with tracing practice. It is satisfactory to note that our results correspond in many ways to those presented by Boyd (1960). We differ mainly in recognizing an intermediate type of fibre, and in maintaining that branching fibres may reunite. It is possible that the rare branching of nuclear-bag fibres noted by Boyd occurred in fibres that we would classify as intermediate.

While the bimodal distribution of the diameters of intrafusal muscle fibres is now firmly established (see e.g. Walker, 1958; Boyd, 1960; Swett & Eldred, 1960), it is significant that Cuajunco (1927, 1940) recognizes fibres of large, medium, and small size in spindles from adult pig and human biceps brachii. In his earlier study of the pig he gives fibre diameters of 18$\mu$ large, 12$\mu$ medium, and 6$\mu$ small (adult, polar measurements), but in his later human study he records the diameters of large and small fibres only, noting that 'the intermediate group varies between these two extremes' (p. 123). He found that the large fibres were the first to arise in development; that the medium fibres arose from somewhat younger myoblasts grouped with the first; and that the small fibres were added later, either by the splitting of the original cells, or by the incorporation of new myoblasts. It seems probable that the additional intrafusal fibres are recruited from cells surrounding the large fibres in much the same way that the number of extrafusal fibres is generally held to increase in the later stages of development by new fibres arising in the neighbourhood of primary muscle fibres to form 'primary' muscle bundles (see J. D. Boyd, 1960). In fact, if the accounts of Tello (1922) and Cuajunco (1927, 1940) of spindle development are related to knowledge of extrafusal muscle fibre development, we may regard the intrafusal bundle as retaining many of the characteristics of a 'primary' extrafusal bundle in consequence of the retardation of development that follows receipt of the primary afferent innervation. The branching of small and intermediate fibres that occurs in some adult spindles is presumably the result of fibres which, though arising from new cells, nevertheless bifurcate during their subsequent growth. The factors which result in such branching in intrafusal bundles but not, apparently, in extrafusal ones, remain to be elucidated in future accounts of muscle development.

An attempt to relate the innervation of the muscle spindle with the individual fibres in the intrafusal bundle has been made by Boyd (1958, 1959). He maintains that the large muscle fibres are innervated by medium-sized efferents which form discrete end-plates of the typical extrafusal type, while the small muscle fibres are innervated by small efferents,

which form plates that are smaller and more variable in form. He has established the efferent nature of another type of ending which assumes the form of a diffuse and often extensive network; this is also, according to him, located on the small muscle fibres. With regard to the afferent innervation, Boyd maintains that while the primary ending is common to both large and small muscle fibres, the secondary ending is located almost exclusively on the latter. Finally, he distinguishes between (1), 'simple' spindles with muscle fibres similar in diameter innervated by a primary ending and discrete end-plates, and (2), 'compound' spindles in which the small muscle fibres, innervated as he describes, are combined with the large. In this scheme there is thus provision for the two types of efferent behaviour of the spindle, whether these are both attributable to efferent fibres within the $\gamma$ range (Pascoe, 1958; Boyd, 1959; Boyd & Davey, 1959), or whether fast $\alpha$ fibres (Granit, Pompeiano & Waltmann, 1959) also play a part. It remains to be seen, however, whether further histological work will confirm this analysis. In the mean time the following points suggest that it may be premature to accept it: (i) While large muscle fibres are always present in the intrafusal bundle, many spindles occur in which medium-sized efferents, as judged by intramuscular diameter, play no part in the innervation (M. N. Adal & D. Barker, unpublished). (ii) In rectus femoris spindles, small muscle fibres are always present in the intrafusal bundle, and bimodality of muscle fibre diameter is always evident. (iii) If the properties of fast and slow muscle fibres may be related to their fibrillar and areal pattern in transverse section, as Krüger (1952) maintains, and if the supposition is correct that there are 'twitch' and 'tonic' mechanisms in the spindle, then one might expect the intrafusal muscle bundle to consist of a combination of wholly fibrillar and areal fibres, but this is not so. Each fibre is both areal and fibrillar in varying proportions, but always predominantly fibrillar. Moreover, the application of Krüger's theory of mammalian muscle does not agree with physiological evidence (see Hunt & Kuffler, 1951), and Eyzaguirre (1960) has failed to detect intrafusal responses in the cat which had the characteristics of slow fibres. (iv) The supposition that nuclear-bag and nuclear-chain fibres constitute separate functional systems must take account of the fact that a nuclear-bag fibre of the intermediate type may bifurcate to produce a nuclear-chain fibre as a side branch. (v) The location of secondary endings almost exclusively on small muscle fibres is doubted by Barker & Ip (1961) in their study of single and tandem spindles in the cat. (vi) Boyd's innervation analysis was made on gold-chloride and methylene blue preparations. In our experience, it is doubtful whether such material is adequate to permit the relation of spindle nerve endings to individual muscle fibres with certainty. We believe

that this information may best come from tracing a number of silver-impregnated spindles in serial transverse section, and this work is in progress.


## SUMMARY

1. The morphology of the intrafusal muscle fibre in the cat was studied mainly in serial transverse sections, the most detailed information being derived from graphic reconstructions of thirty-two spindles. The muscle most frequently used was rectus femoris.

2. The intrafusal bundle of single spindles and tandem spindle units consists of four to twelve muscle fibres of which both length and diameter measurements are bimodal.

3. Large, intermediate, and small types of intrafusal muscle fibres are recognized. The large fibres possess nuclear-bag and myotube regions, and are the longest; they do not branch. Their diameter (12–26$\mu$), and their position in the intrafusal bundle, undergo little change. The intermediate fibres resemble the large but they occasionally branch. Their position tends to change in the intrafusal bundle and their diameter (10–16$\mu$) undergoes considerable variation. The small fibres have a diameter of 4–12$\mu$ and possess nuclear chains in the equatorial region. About one in five branches, and their position in the intrafusal bundle undergoes frequent change.

4. From one to three large fibres (usually two), and from one to eight small fibres (usually four or five) were present in all spindles; intermediate fibres (usually one, occasionally two) were present in about one third.

5. The branching of a small or intermediate fibre takes place over a distance of 60–90$\mu$. With one exception, this always occurred in the proximal pole or in the proximal part of the equatorial region. The two fibres produced may either taper off, or reunite in the distal pole or distal part of the equatorial region. The process of branching or reuniting is quite distinct from the condition where one fibre tapers off alongside another; tapering usually occupies a length of several hundred microns.

6. In the spindles studied all the muscle fibres were predominantly fibrillar, the areal structure occupying a minor proportion of their total length and being most typically present in the juxta-equatorial regions.

7. Reasons are given which suggest that it may be premature to accept Boyd's analysis (1958, 1959) of the innervation of the spindle in terms of the individual muscle fibres composing it.


We wish to express our thanks to Mr Davidson Chi, Mr Jim Tak Charm, and Mr Loung Kar Chuen for technical assistance. The investigation was supported by research grant B-1806 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

## REFERENCES


BARKER, D. (1948). The innervation of the muscle-spindle. *Quart. J. micr. Sci.* **89**, 143–186.

BARKER, D. (1959). Some results of a quantitative histological investigation of stretch receptors in limb muscles of the cat. *J. Physiol.* **149**, 7–9*P*.

BARKER, D. & GIDUMAL, J. L. (1960). Some observations on the morphology of the intrafusal muscle fibre. *J. Physiol.* **153**, 28–29*P*.

BARKER, D. & IP, M. C. (1961). A study of single and tandem types of muscle-spindle in the cat. *Proc. Roy. Soc.* B (in the Press).

BATTEN, F. E. (1897). The muscle-spindle under pathological conditions. *Brain*, **20**, 138–179.

BAUM, J. (1900). Beiträge zur Kenntniss der Muskelspindeln. *Anat. Hefte*, Abt. 1, **13**, (42), 251–305.

BOYD, I. A. (1958). The innervation of mammalian neuromuscular spindles. *J. Physiol.* **140**, 14–15*P*.

BOYD, I. A. (1959). Simple and compound mammalian muscle spindles. *J. Physiol.* **145**, 55–56*P*.

BOYD, I. A. (1960). The diameter and distribution of the nuclear bag and nuclear chain muscle fibres in the muscle spindles of the cat. *J. Physiol.* **153**, 23–24*P*.

BOYD, I. A. & DAVEY, M. R. (1959). $\beta$-efferent fibres in nerves to mammalian skeletal muscle. *J. Physiol.* **149**, 28–29*P*.

BOYD, J. D. (1960). Development of striated muscle. In *Structure and Function of Muscle*, vol. 1, ed. BOURNE, G. H. New York, London: Academic Press.

CILIMBARIS, P. A. (1910). Histologische Untersuchungen über die Muskelspindeln der Augenmuskeln. *Arch. mikr. Anat.* **75**, 692–747.

COOPER, S. (1960). Muscle spindles and other muscle receptors. In *Structure and Function of Muscle*, vol. 1, ed. BOURNE, G. H. New York, London: Academic Press.

CUAJUNCO, F. (1927). Embryology of the neuromuscular spindle. *Contr. Embryol. Carneg. Instn*, **19**, 45–72.

CUAJUNCO, F. (1940). Development of the neuromuscular spindle in human fetuses. *Contr. Embryol. Carneg. Instn*, **28**, 97–128.

DENNY-BROWN, D. (1928). *On the essential mechanism of mammalian posture.* D. Phil. thesis, University of Oxford.

EYZAGUIRRE, C. (1960). The electrical activity of mammalian intrafusal fibres. *J. Physiol.* **150**, 169–185.

FORSTER, L. (1894). Zur Kenntniss der Muskelspindeln. *Virchows Arch.* **137**, 121–153.

GRANIT, R., POMPEIANO, O. & WALTMAN, B. (1959). Fast supraspinal control of mammalian muscle spindles: extra- and intrafusal co-activation. *J. Physiol.* **147**, 385–398.

GRAY, E. G. (1957). The spindle and extrafusal innervation of a frog muscle. *Proc. Roy. Soc.* B, **146**, 416–430.

GRAY, E. G. (1958). The structures of fast and slow muscle fibres in the frog. *J. Anat., Lond.*, **92**, 559–562.

HÄGGQVIST, G. (1960). A study of the histology and histochemistry of the muscle spindles. *Z. Biol.* **112**, 11–26.

HUNT, C. C. & KUFFLER, S. W. (1951). Further study of efferent small-nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* **113**, 283–297.

KRÜGER, P. (1952). *Tetanus und Tonus der quergestreiften Skelettmuskeln der Wirbeltiere und des Menschen.* Leipzig: Akademische Verlagsgesellschaft.

PASCOE, J. E. (1958). Two types of efferent fibres to the muscle spindles of the rabbit. *J. Physiol.* **143**, 54–55*P*.

RUFFINI, A. (1898). On the minute anatomy of the neuromuscular spindles of the cat and their physiological significance. *J. Physiol.* **23**, 190–208.

SHERRINGTON, C. S. (1894). On the anatomical constitution of the nerves of skeletal muscles; with remarks on recurrent fibres in the ventral spinal nerve-root. *J. Physiol.* **17**, 211–258.

SWETT, J. E. & ELDRED, E. (1960). Comparisons in structure of stretch receptors in medial gastrocnemius and soleus muscles of the cat. *Anat. Rec.* **137**, 461–473.

TELLO, J. F. (1922). Die Entstehung der motorischen und sensiblen Nervenendigungen. *Z. ges. Anat.* 1. *Z. Anat. EntwGesch.* **64**, 348–440.

WALKER, L. B. (1958). Diameter spectrum of intrafusal muscle fibres in muscle spindles of the dog. *Anat. Rec.* **130**, 385.

WEISMANN, A. (1861). Ueber das Wachsen der Quergestreiften Muskeln nach Beobachtungen am Frosch. *Z. rat. Med.* **10**, 263–284.

## EXPLANATION OF PLATES

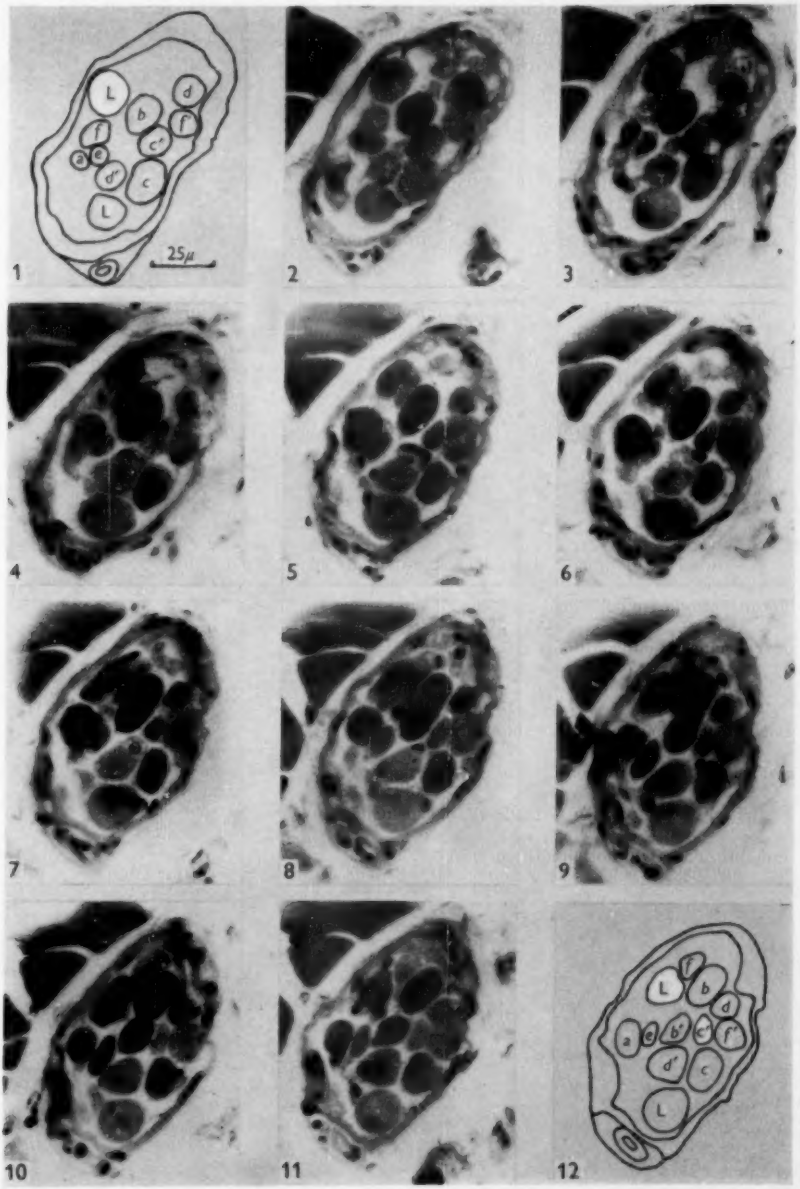
### PLATE 1

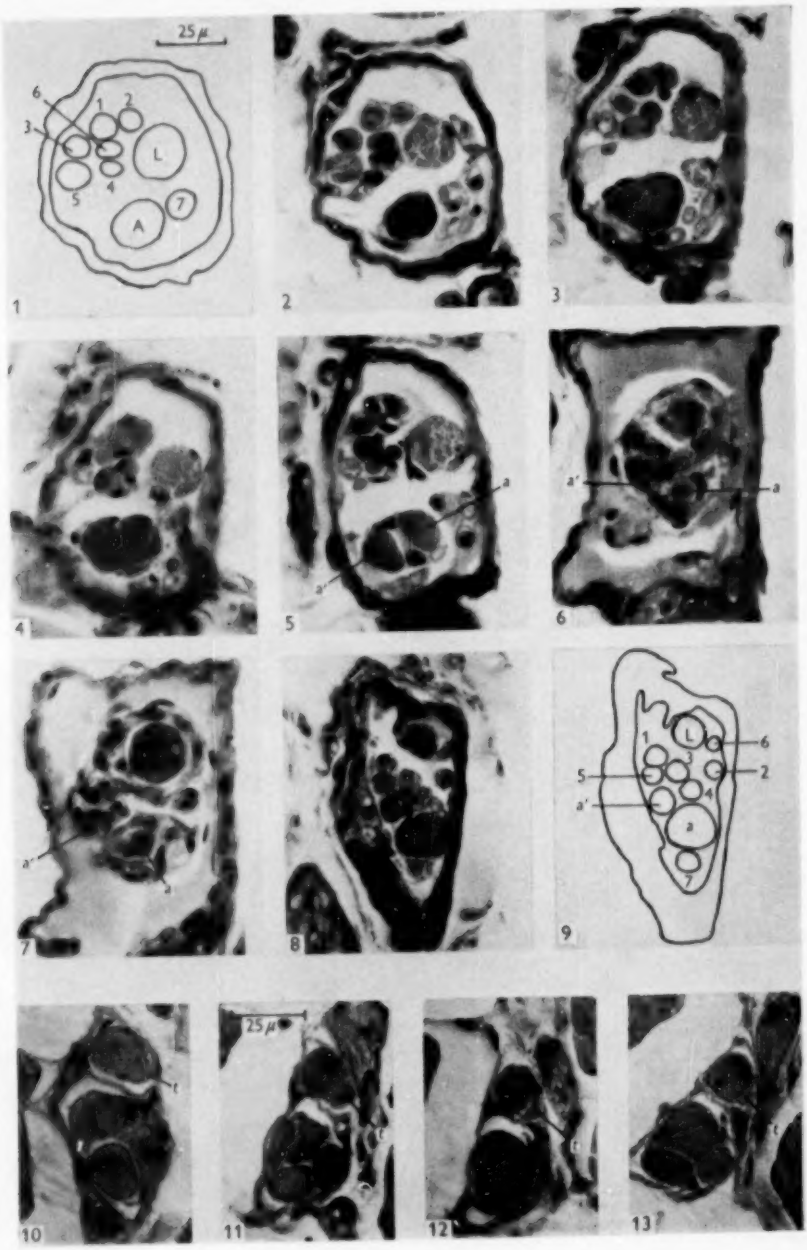
Bifurcation of fibre *b* belonging to the double tandem spindle from cat rectus femoris shown in projection-plan in Text-fig. 4. Figs. 1 and 12 are camera-lucida outline drawings in which the large fibres are indicated by L, and the other fibres are lettered as in Text-fig. 4. Serial transverse 12 $\mu$ paraffin sections stained with haematoxylin and eosin; there is an interval of 3 sections between figs. 1 and 2, and between 4 and 5; 5 sections between figs. 3 and 4; and 6 sections between figs. 2 and 3. Figs. 5–12 represent serial sections. In fig. 9 a crack is present in the bifurcating fibre *b* as an artifact just above the region of constriction. Note (i) fluctuation in diameter of the intermediate fibre *a*; (ii) fibrillar pattern of, e.g. fibres *a* and *b* as compared with areal pattern of, e.g. fibres *d'* and *f'*; (iii) change in structural pattern of fibre *d* from areal in fig. 2 to fibrillar in fig. 11.

### PLATE 2

Figs. 1–9. Bifurcation of fibre A belonging to the single spindle from cat rectus femoris shown in Text-fig. 2. Figs. 1 and 9 are camera-lucida outline drawings of the sections illustrated in figs. 2 and 8 respectively, and the fibres are lettered and numbered as in Text-fig. 2. Serial transverse 12 $\mu$ paraffin sections stained with haematoxylin and eosin; there is an interval of 6 sections between figs. 2 and 3; 2 sections between figs. 4 and 5; 75 sections between figs. 5 and 6; 8 sections between figs. 6 and 7; and 38 sections between figs. 7 and 8. Figs. 3 and 4 represent adjacent sections. Note (i) equatorial nucleation of fibres L, *a*, and *a'* in figs. 6 and 7; (ii) narrower width of the intrafusal bundle and the fibres composing it in fig. 6; (iii) areal pattern of fibre L in fig. 2 as compared with its fibrillar pattern in fig. 8.

Figs. 10–13. Tapering of a fibre *t* alongside a longer fibre which does not alter in diameter. Sections and staining as above; there are successive intervals of 336 $\mu$, 120 $\mu$, and 60 $\mu$ respectively between figs. 10 and 11, 11 and 12, and 12 and 13. Proximal pole of single spindle from cat rectus femoris.


L
b
d
f
a
e
c'
d'
c
L
25μ
1
2
3
4
5
6
7
8
9
10
11
12




D. BARKER AND JANKI L. GIDUMAL

*J. Physiol.* (1961), **157**, *pp.* 529–541
*With 6 text-figures*
*Printed in Great Britain*



# COMPETITION FOR TRIIODOTHYRONINE BETWEEN SERUM PROTEINS AND RED CELLS

BY N. B. MYANT AND C. OSORIO

*From the Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12*

(*Received 6 February* 1961)

It has been shown that the uptake of radioactive triiodothyronine (T3) by red cells suspended in plasma can be used as a diagnostic test for thyrotoxicosis (Hamolsky, 1955). If radioactive T3 is added to a suspension containing equal volumes of normal red cells and normal plasma, about 10% of the T3 becomes attached to the cells, If, however, the serum is taken from a thyrotoxic person (thyrotoxic serum) the uptake may reach 20%. In myxoedema, on the other hand, the uptake may fall to less than 5%. Since the uptake of T3 by red cells has been shown to be due to reversible adsorption (Crispell & Coleman, 1956) it is generally assumed that the proportion taken up is determined by competition between binding sites on the red cells and those on the proteins in the serum. As the high uptake of T3 in thyrotoxicosis is due to an abnormality in the serum and not in the red cells, Hamolsky (1955) concluded that in thyrotoxic serum the binding power for T3 is diminished.

T3 and thyroxine (T4) added to serum at low concentrations are bound by the same thyroxine-binding protein (TBP) (Deiss, Albright & Larson, 1953); TBP has also been shown to bind T4 more strongly than T3 (Larson & Albright, 1955). It might, therefore, seem reasonable to suppose that the rise in the concentration of thyroxine that occurs in thyrotoxicosis would reduce the amount of T3 capable of being bound by the serum, and so lead to an increase in the uptake of T3 by the red cells. However, several workers have rejected this explanation of the red cell test on the grounds that normal serum enriched with T4 does not behave like thyrotoxic serum when tested with rat diaphragm (Hamolsky, Ellison & Freedberg, 1957) or red cells (Richards, Dowling & Ingbar, 1959). In the hope of gaining a better understanding of the red cell test, we have studied the binding of T3 by serum submitted to paper electrophoresis at different hydrogen-ion concentrations, including a pH closer to the physiological than that generally used for paper electrophoresis. We have also compared the binding of T3 and T4 by red cells.

## METHODS

The methods used for the paper electrophoresis, for preparing the buffers and for scanning radioactivity on the paper strips have been described elsewhere (Myant & Osorio, 1960*a*, *b*). When possible, the amount of T3 in each zone of the paper strip was estimated from the concentration in the serum and the proportion of the total activity at each peak recorded by the scanner. The binding capacity of TBP for T4 in tris-maleate at pH 8·6 was estimated from the amount of $^{131}$I-labelled T4 present in the inter-alpha zone after electrophoresis of serum to which enough labelled T4 (100 $\mu$g/100 ml.) had been added to saturate the binding sites on TBP. The samples of normal serum were obtained from two normal adult males. For comparison of the binding of radioactive T4 and T3 by red cells, blood was drawn into a heparinized syringe and the red cells were washed six times with physiological saline after removal of the white cells. A stock suspension was made up to contain 2·5 ml. red cells in 20 ml. Krebs's bicarbonate buffer at pH 7·4. Samples, each of 2·0 ml., were incubated with the radioactive hormone for 2 hr at 37° C in stoppered tubes with gentle shaking. At the end of the incubation the red cells were separated by centrifugation at about 2000 *g* for 20 min. A sample was taken from the supernatant for measurement of radioactivity and the amount of activity in the whole supernatant was estimated from the activity per unit volume and the volume of buffer in the suspension. Radioactivity in the unwashed red cells was estimated as the difference between the total added and that remaining in the supernatant at the end of the incubation. The $^{131}$I-labelled L-triiodothyronine and L-thyroxine, of specific activity 20–30 $\mu$c/$\mu$g, were obtained from Abbott Laboratories Pacific Ltd., Chicago.

## RESULTS

### *Electrophoretic distribution of radioactive triiodothyronine at increasing concentrations*

The binding of radioactive T3 by the protein fractions of serum was studied in samples of normal human serum submitted to paper electrophoresis in tris-maleate buffer at pH 7·6, 8·6 and 9·2, and in 5,5-diethylbarbiturate buffer at pH 8·6. The concentration of added T3 was varied from about 1$\mu$g/100 ml. to 300 $\mu$g/100 ml. serum. Since the distribution of activity in the presence of tris-maleate was markedly influenced by the pH of the buffer, the results obtained at each hydrogen ion concentration will be described separately.

*At pH* 7·6. At the lowest concentration of T3, most of the activity was confined to a single peak in the inter-alpha zone (Fig. 1*a*). The remainder of the activity was distributed between the origin and the posterior limb of the inter-alpha peak, with small humps corresponding roughly with the $\alpha_2$ and $\beta$ bands. There was no activity in the albumin band. As the concentration was raised (Fig. 1*b*), the activity behind the inter-alpha zone increased at the expense of the inter-alpha peak. At a concentration of 100 $\mu$g/100 ml. the main peak corresponded to the $\beta$ band, but at higher concentrations most of the activity was at the origin. Even at the highest concentration tested there was no peak of activity in the albumin or pre-albumin zones.

Since the inter-alpha peak was never clearly separated from the peaks in the $\alpha_2$ and $\beta$ zones, it was difficult to estimate the amount of T3 carried in each zone. As a rough approximation, the amount in the inter-alpha zone was determined from the area that lay under the curve and

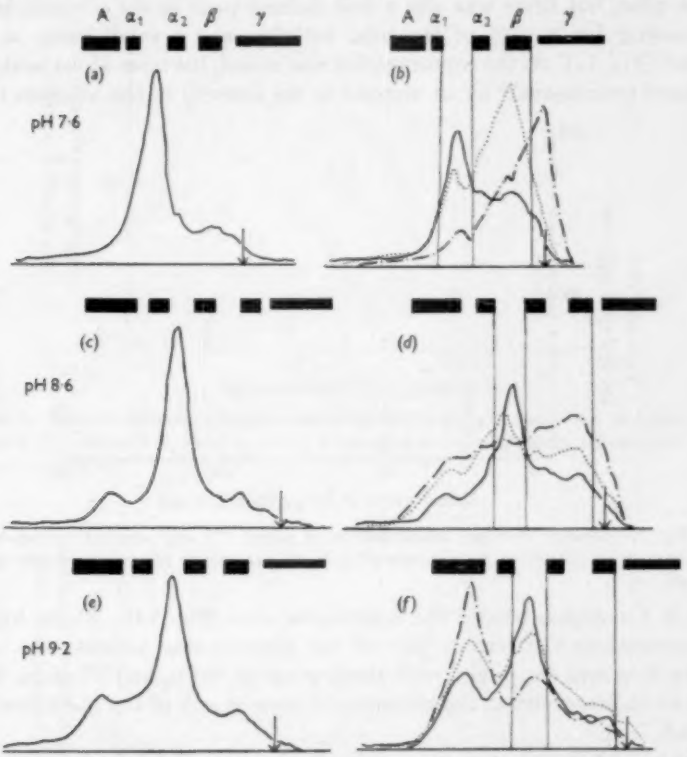



Fig. 1. Distribution of radioactivity along paper strips after electrophoresis of serum in tris-maleate buffer at pH 7·6, 8·6 and 9·2. *a*, *c* and *e*, radioactive T3 added at concentration of 1 $\mu$g/100 ml. serum. *b*, *d* and *f*, T3 added at (25 ——), 100 (.....), and 200 (— · — · —) $\mu$g/100 ml. serum. Arrows show line of application of sample. Solid blocks show positions of protein bands (A = albumin).

was limited by the vertical lines shown in Fig. 1*b*; the amount in the $\alpha_2$ and $\beta$ zones, including the intervening region ($\alpha_2$-$\beta$ zone), was determined in a similar way (see Fig. 1*b*). Figure 2 shows how the amount of T3 carried in the inter-alpha zone, calculated on this basis, varied with the concentration. The amount reached a maximum of about 25 $\mu$g/100 ml. serum when the concentration was 100 $\mu$g/100 ml. At concentrations up to 200 $\mu$g/100 ml. almost all the T3 not carried in the inter-alpha zone

was carried in the $\alpha_2$-$\beta$ zone. At higher concentrations the binding by proteins in this zone approached saturation, since an increasing proportion of the total activity remained at the origin.

*At pH* 8·6. At the lowest concentration the main peak was in the inter-alpha zone, but there was also a well defined peak in the albumin band, accounting for 5–10% of the total activity, and a small hump in the $\beta$ band (Fig. 1*c*). As the concentration was raised, the inter-alpha peak was replaced progressively by an increase in the activity in the albumin band

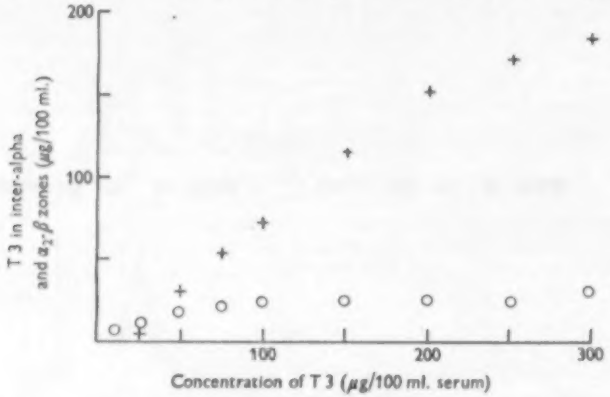



Fig. 2. Relation between concentration of added T3 and amount carried in inter-alpha (○) and $\alpha_2$-$\beta$ (+) zones after electrophoresis in tris-maleate buffer at pH 7·6.

and in the region behind the inter-alpha zone (Fig. 1*d*). At the highest concentrations the greater part of the activity was between the inter-alpha zone and the origin, with small peaks in the $\alpha_2$ and $\beta$ bands. There was no radioactivity in the pre-albumin zone at any of the concentrations tested.

At pH 8·6 it was even more difficult than at pH 7·6 to determine the proportions of the total activity present in different zones along the paper. However, an attempt was made to determine the amounts in the inter-alpha, albumin and $\alpha_2$-$\beta$ zones by dividing up the area under the curve in the way described above. Calculated on this basis the amount of T3 in the inter-alpha zone approached a maximum value of about 35 $\mu$g/100 ml. serum (Fig. 3); that in the albumin increased throughout the range of concentration tested, but the curve suggested an approach to a maximum value of less than 60 $\mu$g/100 ml. The amount in the $\alpha_2$-$\beta$ zone rose steeply to nearly 200 $\mu$g/100 ml. without approaching a plateau at the highest concentration.

*At pH* 9·2. At the lowest concentration the proportion of the total

activity in the albumin band was greater than at pH 8·6. As the concentration was raised, activity was progressively displaced from the inter-alpha zone to the albumin band and, to a smaller extent, to the $\alpha_2$-$\beta$ zone (Fig. 1*e*, *f*). The amount carried in the inter-alpha zone, calculated by the

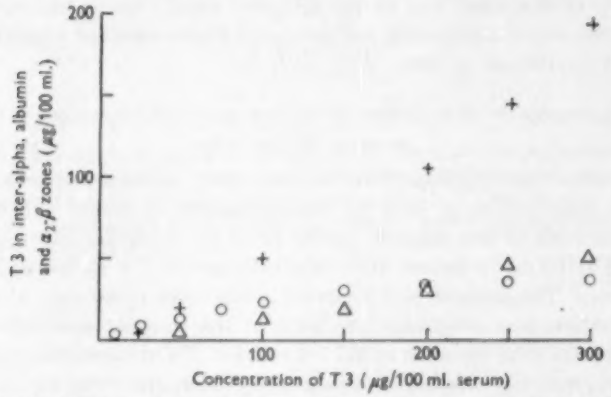



**Fig. 3.** Relation between concentration of added T3 and amount carried in inter-alpha (○), albumin (△) and $\alpha_2$-$\beta$ (+) zones after electrophoresis in tris-maleate buffer at pH 8·6.

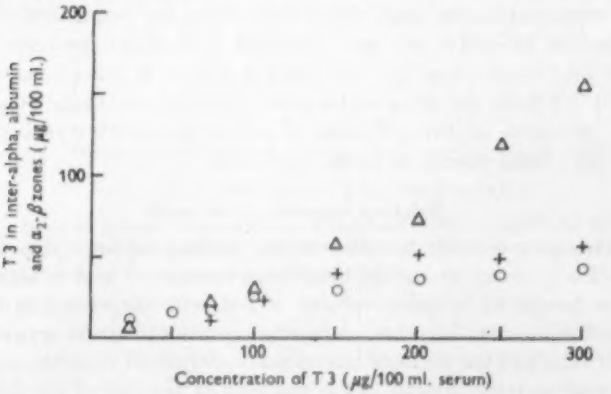



**Fig. 4.** Relation between concentration of added T3 and amount carried in inter-alpha (○), albumin (△) and $\alpha_2$-$\beta$ (+) zones after electrophoresis in tris-maleate buffer at pH 9·2.

method used for deriving Fig. 3, increased to a value of about 40 $\mu$g/100 ml. at a concentration of 300 $\mu$g/100 ml., without reaching a clearly defined plateau (Fig. 4). The amount in the albumin rose steeply to a value of more than 150 $\mu$g/100 ml.

The distribution of T 3 in 5,5-diethylbarbiturate buffer at pH 8·6 was more or less the same as that observed in tris-maleate at pH 7·6. As the concentration was raised, radioactivity was displaced from the inter-alpha zone to the $\alpha_2$-$\beta$ zone. Even at the highest concentration not more than 5% of the total was in the albumin band. As in tris-maleate at pH 7·6, the amount carried in the inter-alpha zone reached a maximum of about 25 $\mu$g/100 ml. serum.

*Electrophoretic distribution of radioactive triiodothyronine in the presence of thyroxine*

In order to study competition for the serum proteins between T 3 and T 4, the distribution of activity was measured in serum submitted to electrophoresis in tris-maleate buffer after the addition of 1 $\mu$g radioactive T 3/100 ml. together with non-radioactive T 4 at increasing concentrations. The amount of T 3 carried in the inter-alpha zone at each T 4 concentration was determined roughly in the manner described above.

The results were the same at pH 7·6 and 8·6. T 4 at increasing concentrations progressively displaced radioactivity from the inter-alpha zone to the region between the $\alpha_2$ band and the origin, no activity appearing in the albumin band (Fig. 5). Some displacement of T 3 was clearly detectable when the concentration of added T 4 was 2 $\mu$g/100 ml. Less than half the T 3 remained in the inter-alpha zone when the concentration of T 4 was raised to 25 $\mu$g/100 ml. and less than 1/10 when the concentration was 100 $\mu$g/100 ml. (Fig. 6). At pH 9·2 T 4 at a given concentration displaced T 3 from the inter-alpha zone to about the same extent as at pH 8·6. However, at this pH some of the radioactivity was displaced to the albumin band as well as to the $\alpha_2$-$\beta$ zone.

*Binding capacity of red cells*

An attempt was made to estimate the binding capacity of red cells for T 3 and T 4 by determining the maximum amount of each substance that could be bound by a given volume of red cells suspended in buffer at pH 7·4. Radioactive hormone at increasing concentrations was incubated with red cells and the amount bound was determined from the proportion of the total activity present in the red cells at the end of the incubation. The proportion of the T 3 and T 4 bound remained more or less constant over a range of concentration from 1 to 10,000 $\mu$g/100 ml. buffer (Table 1), the amount bound increasing linearly as the concentration increased. At a given concentration rather more T 3 than T 4 was bound to the cells; at the highest concentration tested 1 g red cells bound about 620 $\mu$g T 3 and about 510 $\mu$g T 4. Owing to the low solubilities of both hormones at pH 7·4, binding could not be tested at concentrations approaching that

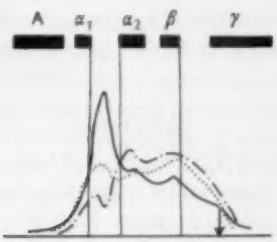



Fig. 5. Distribution of radioactivity along paper strips after electrophoresis of serum in tris-maleate at pH 7·6 with radioactive T3 (1 μg/100 ml. serum) and non-radioactive T4 at concentrations of 10 (———), 25 (.....) and 100 (— — —) μg/100 ml. Arrow shows line of application of sample. Solid blocks show positions of protein bands (A = albumin).

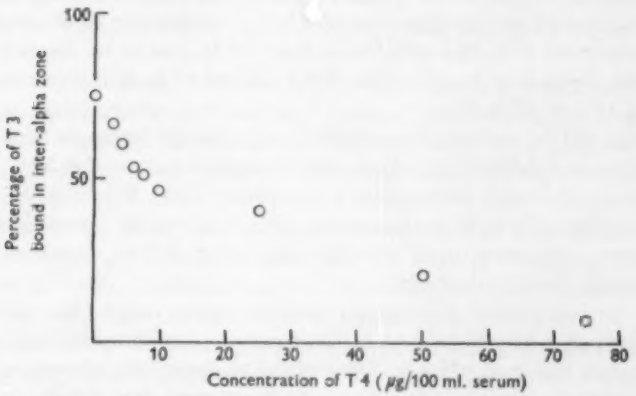



Fig. 6. Relation between concentration of radioactive T3 (1 μg/100 ml. serum) carried in inter-alpha zone and concentration of added T4 in serum submitted to paper electrophoresis in tris-maleate buffer at pH 7·6.

TABLE 1. Uptake of T3 and T4 by normal red blood cells. 0·25 ml. R.B.C. + 2 ml. Krebs's bicarbonate buffer (pH 7·4) containing radioactive T3 or T4 at different concentrations incubated for 2 hr at 37°

| Concentration of hormone (μg/100 ml. buffer) at start of incubation | % activity in R.B.C. at end of incubation | | Hormone bound (μg/g R.B.C.) | |
|---|---|---|---|---|
| | T3 | T4 | T3 | T4 |
| 1 | 78·9 | 61·4 | 0·06 | 0·05 |
| 100 | 78·6 | 61·0 | 6 | 5 |
| 500 | 80·3 | 62·4 | 32 | 25 |
| 1000 | 77·1 | 64·7 | 62 | 52 |
| 5000 | 78·3 | 64·4 | 313 | 258 |
| 10,000 | 77·3 | 63·6 | 618 | 509 |

required to saturate the binding sites. Binding capacity could not, therefore, be determined in conditions comparable with those used in the red cell test, though the results of Table 1 give a minimum value.

## DISCUSSION

The binding capacity of TBP for T 4, determined from the maximum amount carried in the inter-alpha zone, has been found to lie between 15 and 30 $\mu$g/100 ml. serum in most normal people (Robbins & Rall, 1957; Tanaka & Starr, 1959). The T 4-binding capacity of the samples of normal serum used in this work was between 25 and 30 $\mu$g/100 ml. when measured by electrophoresis in tris-maleate at pH 8·6. In view of the errors in determining the amount of T 3 carried in the inter-alpha zone, it is reasonable to conclude, therefore, that at pH 8·6 TBP has about the same binding capacity (in moles of hormone/ml. serum) for T 3 as for T 4. This suggests that all the binding sites on TBP are available to both hormones. It is consistent with this suggestion that T 3 begins to be displaced from the inter-alpha zone by T 4 when T 4 is added at a concentration as low as 2 $\mu$g/100 ml. (Fig. 6).

As the pH is reduced from 9·2 to 7·6 albumin becomes increasingly ineffective in binding T 3, both at low concentrations and at high concentrations approaching that required to saturate TBP. Whatever the reason for this effect of pH, it seems unlikely that binding by albumin can have any direct influence upon the distribution of T 3 between serum and red cells at physiological pH.

The tendency for T 3 to spread into the region behind the inter-alpha zone was noticed by Deiss *et al.* (1953) during electrophoresis in 5,5-diethylbarbiturate buffer at pH 8·6. Some of the radioactivity remaining behind the inter-alpha zone in tris-maleate buffer is probably due to binding of T 3 by the $\alpha_2$- and $\beta$-globulins, since peaks corresponding to these proteins were usually observed. On the other hand, some of it may be due to T 3 bound to the filter paper rather than to any of the serum proteins. For this reason, and also because of the lack of resolution between the peaks behind the inter-alpha zone, an exact estimate of the binding capacity of the $\alpha_2$- and $\beta$-globulins is not possible. Nevertheless, it seems clear that as the pH is reduced, the more slowly moving globulins bind an increasing proportion of the T 3 that becomes available as the binding sites on TBP approach saturation. This may simply be a consequence of the fall in the binding power of albumin. At pH 7·6 an increasing proportion of the radioactivity remains at the origin as the concentration of T 3 is raised above 100 $\mu$g/100 ml. This, as already noted, shows that the $\alpha_2$- and $\beta$-globulins approach saturation at the highest concentrations tested; Fig. 2

suggests that the sum of the binding capacities of these proteins is not more than 300 $\mu$g/100 ml. serum at pH 7·6.

Our attempt to measure the binding capacity of red cells for T 3 and T 4 was unsuccessful. However, it is possible to draw certain conclusions from the results. When the binding sites on the red cells approach saturation there should be a fall in the proportion of bound to total hormone as the concentration of hormone in the buffer is increased. The fact that no such fall occurs (Table 1) shows that, even at the highest concentration tested, the majority of the binding sites are still free. The binding capacity for both T 3 and T 4 must, therefore, be much greater than 50 mg/100 g. The binding affinity for both hormones, on the other hand, must be very low, since 20 % or more of the total hormone in the red cell suspension is free at the lowest concentration. Table 1 also shows that red cells bind a larger proportion of T 3 than of T 4 at a given concentration of hormone. This is in agreement with the findings of Crispell, Kahana & Hyer (1956) and suggests that the affinity of T 3 for red cells is slightly higher than that of T 4.

Christensen (1960) and Robbins & Rall (1960) have pointed out that a rise in the concentration of T 4 should displace T 3 to the red cells, provided the binding capacity of the serum proteins remains constant. Our observations on the electrophoretic distribution of T 3 help to show how this may occur.

We have shown elsewhere (Myant & Osorio, 1960*b*) that the effect of pH on the binding of T 4 by human serum is such as to suggest that, at hydrogen-ion concentrations near the physiological, TBP and albumin are the only proteins that bind T 4. If this is true, the binding capacity of TBP available to T 3 in normal plasma is approximately equal to the total concentration of binding sites on TBP, minus the concentration of T 4 not bound to albumin. When T 3 is added to a suspension of red cells in plasma it is shared between the red cells and those proteins in the plasma that are capable of binding it at physiological pH. At low concentrations of T 3 the fraction bound by the red cells and by the various binding proteins of the plasma will in each case be roughly proportional to the product of binding affinity and binding capacity available to T 3. Since it seems likely that several of the serum proteins compete for T 3 at physiological pH and that they have different affinities and binding capacities, it is impossible to predict how the partition of T 3 between red cells and plasma would vary with changes in the binding capacity of TBP available to T 3. However, it is possible to predict what should happen in general terms.

Figure 1 shows that at pH 7·6 nearly all the T 3 added to serum at low concentration is bound by TBP. Since the capacity of the $\alpha$- and $\beta$-globulins is greater than that of TBP (Fig. 2), the affinity of TBP for

T 3 must be much greater than that of any other serum protein. As we have pointed out above, the capacity of the red cells is probably much higher than that of all the serum proteins together, whereas their binding affinity is very low. The net result of these factors is that in the presence of equal volumes of normal plasma and red cells about 90% of the T 3 remains in the plasma, almost all of this being bound by TBP. If the concentration of T 4 is increased, there is a fall in the binding capacity of TBP available to T 3 and this results in a displacement of T 3 to the $\alpha$- and $\beta$-globulins (Fig. 5). Since the binding affinity of these globulins is rather low and their capacity is much less than that of the red cells, this displacement of T 3 leads to an increase in the proportion bound by red cells. As the concentration of T 4 is increased, the concentration of binding sites on TBP available to T 3 will approach zero and the proportion of T 3 bound to red cells will approach the value that would be observed if T 3 were shared only between red cells and the $\alpha$- and $\beta$-globulins. The results of Friis (1960, Fig. 3) suggest that this value is between 20 and 25%.

When the red cell test was first described, Hamolsky (1955) observed that if radioactive T 4, instead of T 3, was used, less than 1% was taken up by the red cells and the uptake could not be used as a diagnostic test for thyrotoxicosis. Crispell *et al.* (1956) suggested that this difference was due to the low binding affinity of T 4 for red cells compared with that of T 3. However, the difference in affinity is too small to account for the difference in uptake by the red cells. It seems more likely that the difference in uptake is due to the binding properties of albumin. Whereas albumin has a high binding power for T 4 at a pH close to the physiological (Myant & Osorio, 1960*b*), its power to bind T 3 is almost completely suppressed at pH 7·6 (Fig. 1*a*, *b*). Hence, as the binding sites on TBP become filled, the red cells compete with albumin for T 4, but with the weakly binding globulins for T 3.

Several workers have considered the possibility that the high uptake of T 3 by red cells in thyrotoxicosis is due to an increase in the proportion of binding sites on TBP that are occupied by T 4 (Hamolsky, Golodetz & Freedberg, 1959; Robbins & Rall, 1960; Ingbar & Freinkel, 1960). If the high uptake in thyrotoxicosis is to be explained in this way, it should be possible to raise the uptake in normal plasma by adding enough T 4 to raise the concentration to the level found in thyrotoxicosis. Friis (1960) concluded that T 4 added to plasma *in vitro* does not affect the uptake of T 3 until the concentration of added T 4 exceeds 6 $\mu$g/100 ml. Richards *et al.* (1959) also say that the addition of T 4 to normal plasma has little effect on the uptake of T 3, though their experiments have not been published. It has also been found (Hamolsky *et al.* 1959) that the uptake of T 3 by red cells is increased in plasma from patients with cirrhosis of the

liver or malignant disease, in whom the plasma concentration of thyroxine and the binding capacity of TBP are normal (Ingbar & Freinkel, 1960). Ingbar & Freinkel (1960) have put forward an explanation of the red cell test that takes account of the thyroxine-binding substance moving ahead of albumin during paper electrophoresis in tris-maleate buffer at pH 8·6 (pre-albumin binding substance or TBPA). They have claimed that the binding capacity of this substance, as measured in tris-maleate at pH 8·6, is reduced in thyrotoxicosis and in many non-thyroid diseases. Although TBPA does not bind T 3 (Ingbar, 1958), Ingbar & Freinkel (1960) have pointed out that a fall in the capacity of TBPA could lead to a high uptake of T 3 by red cells because a relatively large proportion of the total T 4 in the serum would be carried on TBP and there would, therefore, be a fall in the concentration of binding sites on TBP available to T 3. In support of this explanation they claim that the rise in T 3 uptake in plasma from thyrotoxic patients and from patients with non-thyroid disease is correlated with a fall in the capacity of TBPA.

Against this explanation, however, our observations on the effect of pH on the binding of T 4 in serum (Myant & Osorio, 1960*b*) suggest that the binding of T 4 by TBPA is suppressed at physiological pH. The failure of Ingbar & Freinkel (1960) to find a consistent increase in T 3 uptake from normal plasma enriched with T 4 is difficult to reconcile with our observation that T 3 is displaced from TBP to the $\alpha_2$- and $\beta$-globulins (Fig. 6) when the concentration of T 4 is raised by not more than 2 $\mu$g/100 ml. Moreover, J. Gartside, A. W. G. Goolden, D. Jackson & C. Osorio (unpublished observations) have shown that the uptake of T 3 by red cells in normal plasma is significantly increased when T 4 is added at a concentration of 1 $\mu$g/100 ml. They have also shown that, at a given concentration of unoccupied binding sites on TBP, the uptake of T 3 in the red cell test is the same in plasma from thyrotoxic and myxoedematous people as in normal plasma enriched with T 4. In order to explain the results of the red cell test in thyroid disease it may not, therefore, be necessary to postulate any change in the serum other than an alteration in the concentration of binding sites on TBP available to T 3. It must be admitted, however, that this does not explain the high T 3 uptake claimed to occur in patients with non-thyroid diseases in whom the total binding capacity of TBP and the serum concentration of thyroxine are both within normal limits (Ingbar & Freinkel, 1960).

SUMMARY

1. The effect of pH on the distribution of radioactive triiodothyronine (T 3) in normal human serum was studied by paper electrophoresis.

2. At low concentrations most of the T 3 was carried in the inter-alpha

zone. As the concentration was increased, T 3 was progressively displaced from the inter-alpha zone. Displacement was mainly to albumin at pH 9·2, to albumin and the $\alpha_2$- and $\beta$-globulins at pH 8·6, and to the $\alpha_2$- and $\beta$-globulins alone at pH 7·6.

3. The T 3-binding capacity of the inter-alpha, albumin and $\alpha_2$-$\beta$ zones was estimated roughly at each pH, from the maximum amount of T 3 bound at each zone. At pH 7·6, the T 3-binding capacity of the inter-alpha zone was about 25 $\mu$g/100 ml. serum; that of the $\alpha_2$-$\beta$ zone was between 200 and 300 $\mu$g/100 ml.; that of albumin was zero.

4. The T 3-binding capacity of red cells was found to be at least 620 $\mu$g/g at pH 7·4.

5. When T 4 was added at increasing concentrations to serum containing radioactive T 3 at pH 7·6, the T 3 was progressively displaced from the inter-alpha zone to the $\alpha_2$- and $\beta$-globulins. Definite displacement was observed when the concentration of added T 4 was 2 $\mu$g/100 ml.

6. It is concluded that when T 3 is added to a mixture of red cells and plasma, the partition of T 3 between red cells and plasma is determined by competition between red cells, the $\alpha_2$- and $\beta$-globulins, and the binding sites on the inter-alpha binding substance that are not already occupied by T 4.

## REFERENCES


Christensen, L. K. (1960). Triiodothyronine uptake by erythrocytes. *Endocrinology*, **66**, 138–140.

Crispell, K. R. & Coleman, J. (1956). A study of the relative binding capacity of plasma proteins, intact human red cells, and human red cell stroma for radioactive I-131-labelled L-thyroxine. *J. clin. Invest.* **35**, 475–480.

Crispell, K. R., Kahana, S. & Hyer, H. (1956). The effect of plasma on the *in vitro* uptake or binding by human red cells of radioactive I-131 labelled L-thyroxine and L-triiodothyronine. *J. clin. Invest.* **35**, 121–124.

Deiss, W. P., Albright, E. C. & Larson, F. (1953). Comparison of *in vitro* serum protein binding of thyroxine and tri-iodo-thyronine. *Proc. Soc. exp. Biol., N.Y.*, **84**, 513–516.

Friis, T. (1960). On the mechanism of the *in vitro* uptake of $^{131}$I-labelled L-triiodothyronine by human erythrocytes. *Acta endocr., Copenhagen*, **33**, 134–141.

Hamolsky, M. W. (1955). The plasma protein-thyroid hormone complex in thyrotoxicosis vs. euthyroidism in man. *J. clin. Invest.* **34**, 914.

Hamolsky, M. W., Ellison, H. E. & Freedberg, A. S. (1957). The thyroid hormone-plasma protein complex in man. 1. Differences in different states of thyroid function. *J. clin. Invest.* **36**, 1486–1497.

Hamolsky, M. W., Golodetz, A. & Freedberg, A. S. (1959). The plasma protein-thyroid hormone complex in man. III. Further studies on the use of the *in vitro* red blood cell uptake of $I^{131}$-l-triiodothyronine as a diagnostic test of thyroid function. *J. clin. Endocrin.* **19**, 103–116.

Ingbar, S. H. (1958). Pre-albumin: a thyroxine-binding protein of human plasma. *Endocrinology*, **63**, 256–259.

Ingbar, S. H. & Freinkel, N. (1960). Regulation of the peripheral metabolism of the thyroid hormones. *Recent Progr. Hormone Res.* **16**, 353–394.

Larson, F. C. & Albright, E. C. (1955). The specificity of thyroxine binding by serum alpha globulin. *Endocrinology*, **56**, 737–741.

Myant, N. B. & Osorio, C. (1960*a*). Observations on filter-paper electrophoresis of thyroxine in barbiturate buffer. *J. Physiol.* **152**, 391–403.

MYANT, N. B. & OSORIO, C. (1960*b*). Paper electrophoresis of thyroxine in tris-maleate buffer. *J. Physiol.* **152**, 601–612.

RICHARDS, J. B., DOWLING, J. T. & INGBAR, S. H. (1959). Alterations in the plasma transport of thyroxine in sick patients and their relation to the abnormality in Graves' disease. *J. clin. Invest.* **38**, 1035.

ROBBINS, J. & RALL, J. E. (1957). The interaction of thyroid hormones and protein in biological fluids. *Recent Progr. Hormone Res.* **13**, 161–202.

ROBBINS, J. & RALL, J. E. (1960). Proteins associated with the thyroid hormones. *Physiol. Rev.* **40**, 415–489.

TANAKA, S. & STARR, P. (1959). The binding of thyroxine analogues by human serum protein. *Acta endocr., Copenhagen*, **31**, 161–170.

*J. Physiol.* (1961), **157**, *pp.* 542–564
*With* 14 *text-figures*
*Printed in Great Britain*

# PARTIAL REVERSAL OF THE EFFECTS OF THYROXINE ON LIPID SYNTHESIS IN RAT LIVER BY THE ADDITION OF COFACTORS *IN VITRO*

BY K. FLETCHER AND N. B. MYANT

*From the Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12

(*Received* 17 *February* 1961)

The synthesis of cholesterol by rat liver slices is increased, and that of fatty acids is depressed, if the rat is injected with thyroxine before removal of the liver (Fletcher & Myant, 1958). Treatment with thyroxine has also been shown to affect the synthesis of lipids in cell-free fractions of liver from which the mitochondria have been removed by centrifugation at 10,000 *g* ($S_{10}$ fractions) (Fletcher & Myant, 1960). In $S_{10}$ fractions cholesterol synthesis is increased after daily injections of 20 $\mu$g thyroxine and is depressed after larger doses; fatty acid synthesis is depressed after all doses of thyroxine from 20 to 100 $\mu$g/day. The depression of lipid synthesis in the $S_{10}$ fractions is associated with a fall in glycogen content. We suggested that in the $S_{10}$ fraction glycogen is the main source of energy for the regeneration of adenosine triphosphate (ATP) and of other cofactors necessary for the synthesis of lipids, and that when lipid synthesis is depressed in $S_{10}$ preparations from thyroxine-treated rats this is because their lack of glycogen leads to a shortage of essential cofactors. In this paper we describe observations which show that glycogen does regenerate ATP in the $S_{10}$ fractions and that the inhibitory effects of thyroxine treatment upon lipid synthesis can be reversed by the addition of ATP and other cofactors *in vitro*.

During the course of this work we made some observations on the relation between ATP concentration and cholesterol synthesis *in vitro* that suggested a possible explanation of the increase in cholesterol synthesis by liver slices from thyroxine-treated rats. This led us to study the optimal concentration of ATP for lipid synthesis in $S_{10}$ fractions from which the endogenous ATP had been removed by dialysis.

This paper also includes observations on the glycogen content of the livers of normal and treated rats. These observations are more detailed than those we have already reported (Fletcher & Myant, 1960), which were based only on the naked-eye appearance of the glycogen pellet obtained by centrifugation.

## METHODS

The rats were albino males of an inbred strain weighing about 150 g. The treated animals were given sodium L-thyroxine daily by intraperitoneal injection for 9 days, the control animals receiving daily injections of the same volume of the fluid used for dissolving the thyroxine. At the end of the period of treatment the rats were killed by a blow on the head and the livers removed. Cell-free fractions were prepared in phosphate buffer (Bucher & McGarrahan, 1956) by the methods described elsewhere (Fletcher & Myant, 1960). For most experiments the $S_{16}$ fraction was used. This fraction contains the supernatant and the particles remaining in suspension after centrifugation for 20 min at 10,000 *g*. For some experiments the $S_{16}$ fraction was spun for 1 hr at 104,000 *g* and the supernatant from this spinning ($S_{164}$) was decanted from the pellet (*M*) containing microsomes and glycogen. When necessary the microsomes were separated from the glycogen by gentle shaking with a little buffer solution. Lipid synthesis was estimated *in vitro* as the amount of 1-$^{14}$C-acetate incorporated into the cholesterol and fatty acids of the liver fractions after incubation for 2 hr at 37° C. The incubations were carried out in a standard medium consisting of the buffer solution used for preparing the fractions, with reduced diphosphopyridine nucleotide (DPNH, 1·0 $\mu$mole), oxidized triphosphopyridine nucleotide (TPN, 0·5 $\mu$mole), glutathione (20 $\mu$mole), and sodium 1-$^{14}$C-acetate (20 $\mu$mole + 5 $\mu$c); other cofactors were added as desired. Each flask contained the equivalent of 0·7 g of fresh liver; the final volume in each flask, including homogenate and buffer, was 3·5 ml. At the end of the incubation cholesterol as digitonide and fatty acids as soaps were isolated from the contents of the flasks and assayed for radio-activity by methods described elsewhere (Fletcher & Myant, 1960). The amounts of lipid synthesized were expressed as micromoles radioactive acetate incorporated into cholesterol or fatty acids per gram of fresh liver.

For each experiment on the effect of adding cofactors the $S_{16}$ fraction was prepared from the pooled livers of six treated rats (treated preparation) and of six controls (control preparation), matched for weight with the treated animals. Synthesis of lipids was then measured in incubation flasks, usually paired, with or without the addition of cofactors. The livers from six rats usually gave enough $S_{16}$ fraction for more than 20 flasks. The values observed within each pair of incubation flasks differed from each other by less than 10 % in almost all cases.

*Estimation of glycogen.* The liver was frozen in liquid air immediately after removal from the rat. A portion of the frozen liver was then weighed and dropped into KOH solution, 33 g/100 ml., at 100°. After digestion on the steam-bath for 1 hr, glycogen was isolated by the method of Somogyi (1957) and hydrolysed to glucose by digesting it in boiling 4 N-$H_2SO_4$ for 1 hr. The glucose was then estimated by the method of Nelson (1944), with the Somogyi reagent (Somogyi, 1945).

*Estimation of ATP.* The liver was dropped into liquid air immediately after removal from the rat. Portions of about 200 mg were crushed to a fine powder in a cooled steel cylinder. A weighed quantity of the powder was then stirred into a few millilitres of ice-cold 10 % trichloroacetic acid and left for 10 min. The mixture was neutralized with NaOH, made up to 10 ml. with the glycine buffer used for the assay, and diluted with buffer to give a final concentration of about 1 $\mu$g ATP/ml. The ATP was estimated from the light emitted by the fire-fly enzyme system, by a modification of the method of Strehler & Totter (1954). About 50 mg dried fire-fly tails (Schwarz Bioresearch Inc., New York) were ground with glass powder in 10 ml. of 0·1 M arsenate buffer at pH 7·2 and left at −15° C until required. On the day of the assay the mixture was thawed and spun at 700 *g* for 10 min at 5° C. The slightly turbid supernatant, containing the enzyme system, was decanted and kept on ice during the assay. Samples of the liver extracts (0·04–0·1 ml.) to be assayed were added to a solution containing 2 ml. 0·05 M glycine buffer at pH 7·2, 0·2 ml. 0·1 M-$MgSO_4$ and 0·2 ml. of enzyme solution in 3 ml. cuvettes housed in a light-tight box placed against the end of a photo-

multiplier tube (EMI-6097/5). This was connected to a potentiometer recorder. The deflexion was read at 20 sec after addition of the ATP. The response of the recorder was linear over a range of ATP from 0·01 to 0·1 $\mu$g; there was no response to adenosine diphosphate. Standard curves were prepared for each set of assays on any day.

The avidin was prepared from fresh hens' eggs by the method of Woolley & Longsworth (1942).

## RESULTS

### *Concentration of glycogen in whole liver*

In an earlier part of this work (Fletcher & Myant, 1960), thyroxine treatment was shown to cause a fall in the concentration of glycogen in the liver. However, this finding was based on the naked-eye appearance of the pellet obtained by centrifugation of the $S_{10}$ at 104,000 $g$. In view of the probable importance of glycogen as an energy source for ATP regeneration in the $S_{10}$, it seemed desirable to obtain more accurate information about the glycogen content of the livers of treated rats, and to

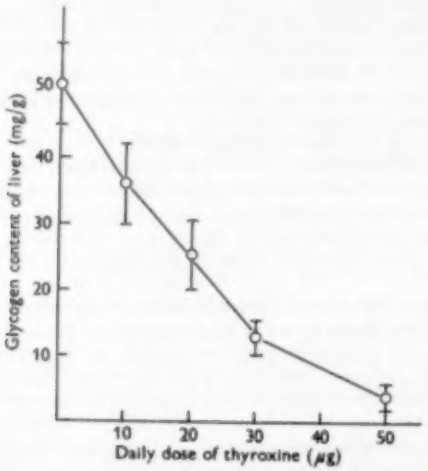



Fig. 1. Concentration of glycogen (mg/g wet weight) in livers of normal and thyroxine-treated rats. Vertical lines show S.E. of mean.

see how this was related to the dose of thyroxine. Figure 1 shows the glycogen concentration in the livers of normal rats and of rats given doses of thyroxine ranging from 10 to 50 $\mu$g/day. In the normal livers the concentration averaged $51 \pm 6{\cdot}1$ mg/g wet weight. After treatment with 10 $\mu$g thyroxine/day there was a significant fall in the glycogen concentration and after 50 $\mu$g/day the level was less than 10 % of that in the controls.

*Concentration of ATP in whole liver and $S_{10}$ fractions*

The ATP content of the livers and of the pooled $S_{10}$ fraction was measured in groups of control and treated rats. In the whole livers from normal rats the average concentration of ATP was $2{\cdot}4 \pm 0{\cdot}2$ $\mu$mole/g wet weight. After treatment with increasing doses of thyroxine there was a progressive fall in ATP concentration to an average value of $1{\cdot}0 \pm 0{\cdot}1$ $\mu$mole/g when the daily dose was 50 $\mu$g (Fig. 2). In the $S_{10}$ fraction from normal rats the average concentration of ATP was 0·65 $\mu$mole/ml. After treatment with 50 $\mu$g thyroxine/day the concentration fell to an average of 0·31 $\mu$mole/ml.

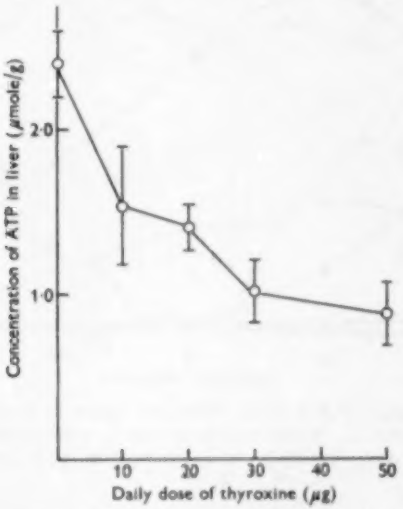



Fig. 2. Concentration of ATP ($\mu$mole/g wet weight) in livers of normal and thyroxine-treated rats. Vertical lines show S.E. of mean.

*Glycogen as an energy source for ATP regeneration in the $S_{10}$ fraction*

If glycogen is the main source of energy for the regeneration of ATP in the $S_{10}$, ATP might be expected to disappear more rapidly from the treated than from the control preparations. The rates of disappearance of endogenous and exogenous ATP were therefore compared in treated and control preparations. $S_{10}$ fractions were prepared from normal rats and from rats given 50 $\mu$g thyroxine/day and were then incubated in the standard medium, with and without added ATP. Samples were taken at zero time, and then at various intervals, for estimation of ATP. Figure 3 shows the results of a typical experiment. When no exogenous ATP was

added, the ATP concentration in the control preparation rose slightly during the first 30 min and then fell to less than 0·10 $\mu$mole/ml. by the end of the second hour; in the treated preparation the initial concentration was less than that in the control and the rate of disappearance was greater, the concentration falling almost to zero during the first 15 min. When exogenous ATP (2 $\mu$mole/ml.) was added, the rate of disappearance in the treated preparation was much greater than that in the control.

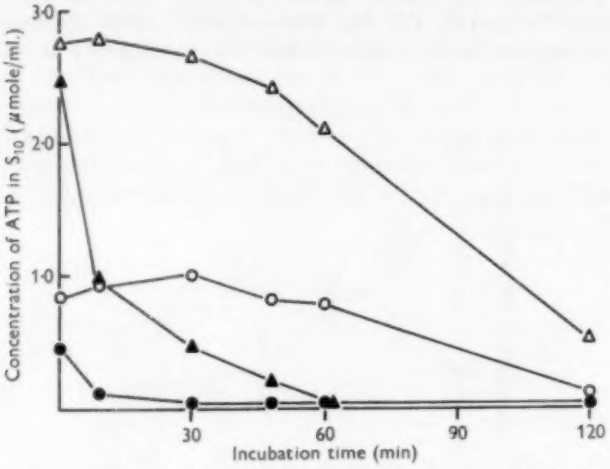



Fig. 3. Concentration of ATP in $S_{10}$ during incubation in standard medium at 37° C for 2 hr. Control (Δ—Δ) and treated (▲—▲) $S_{10}$ to which ATP (2 $\mu$mole/ml.) was added at zero time; control (○—○) and treated (●—●) $S_{10}$ with no exogenous ATP.

The high rate of disappearance in the treated $S_{10}$ fraction with added ATP was such that by the end of the first half hour the concentration was lower than that in the control without added ATP.

Further evidence that glycogen acts as a source of energy for the formation of ATP was obtained by studying the effect of exogenous glycogen on the rate of disappearance of ATP during a 2 hr incubation period. Treated and control $S_{10}$ fractions were prepared in the usual way. The treated fraction was then divided into two parts. To one, glycogen was added at zero time in sufficient quantity to raise the concentration approximately to that in the control preparation; to the other, glycogen was added 1 hr after the beginning of the incubation. The control preparation received no exogenous glycogen. When the glycogen was added to the treated preparation at zero time, the curve of disappearance of ATP was restored almost to that seen in the control preparation (Fig. 4). When the

glycogen was withheld until the end of the first hour, the ATP concentration fell to zero within the first 45 min. Within 15 min of the addition of glycogen, ATP reappeared, rising to a maximum of 1·4 μmole/ml. and then declining. In the treated preparation to which glycogen was not added at the beginning of the incubation, synthesis of cholesterol and fatty acids showed a marked slowing up during the second half hour, followed by a secondary rise after the addition of glycogen (Fig. 4).

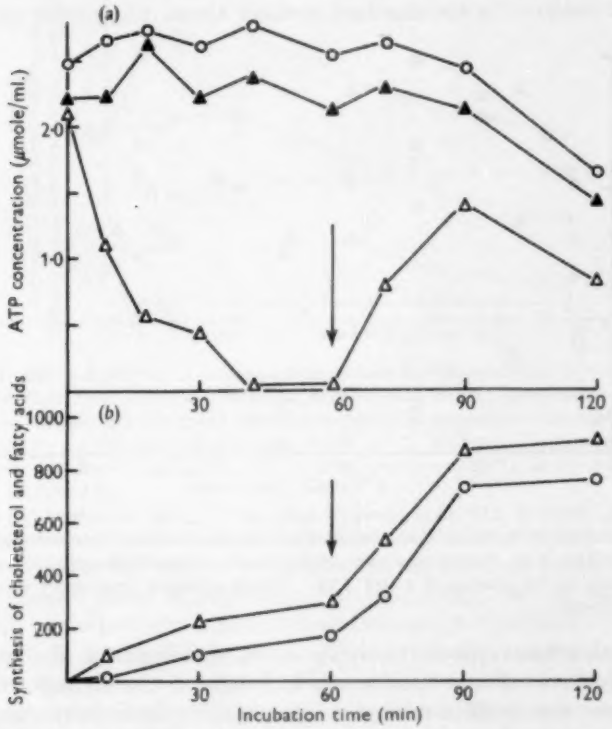



Fig. 4. Effect of glycogen on concentration of ATP and on lipid synthesis in $S_{10}$ during incubation in standard medium at 37 °C for 2 hr. (*a*) ATP concentration in control $S_{10}$ (○—○), in treated $S_{10}$ to which glycogen (30 mg/ml.) was added at zero time (▲—▲) and in treated $S_{10}$ with glycogen (10 mg/ml.) added at end of 1 hr (Δ—Δ). Exogenous ATP (2 μmole/ml.) was added to all three fractions at zero time. (*b*) $^{14}$C-acetate (counts/min from digitonide or soap at infinite thickness) incorporated into cholesterol (○—○) and fatty acids (△—△) in the treated $S_{10}$ to which glycogen was added at end of first hour. Arrow shows addition of glycogen.

*Effect of adding ATP to* $S_{10}$

Preliminary experiments showed that when ATP was added to the control preparations incubated for 2 hr in the standard medium, synthesis of fatty acids was increased and that of cholesterol was reduced. A more detailed study of these effects (Fig. 5) showed that as the concentration of added ATP was raised, fatty acid synthesis increased to a maximum at between 2 and 4 $\mu$mole ATP/ml. and then declined, eventually falling to the level observed in the standard medium alone. Cholesterol synthesis,

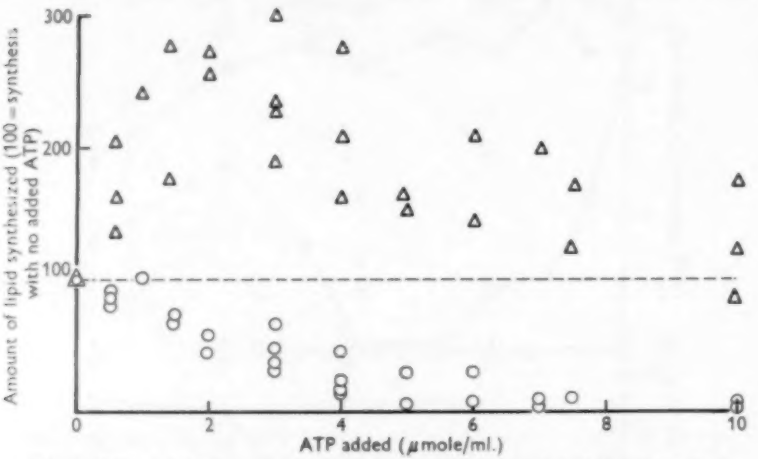



Fig. 5. Effect of ATP on synthesis of fatty acids (△) and cholesterol (○) from 1-$^{14}$C-acetate in $S_{10}$ fraction from normal rat liver incubated in standard medium at 37 °C for 2 hr. Values represent $\mu$mole acetate incorporated, expressed as % synthesis in the absence of added ATP. Results collected from many different experiments.

on the other hand, fell continuously as the concentration of added ATP was raised from 0·5 to 10·0 $\mu$mole/ml. In contrast to the findings with fatty acids, there was no initial rise to a maximum at low concentrations of ATP.

The response of the treated preparations to ATP depended to some extent on the dose of thyroxine given to the rats. After treatment with 30-$\mu$g doses of thyroxine, ATP usually stimulated lipid synthesis, as shown in Fig. 6. In this experiment the amounts of fatty acid and cholesterol synthesized in the standard medium alone were 17 and 65 %, respectively, of the amounts synthesized in the control preparation. As the concentration of added ATP was raised from 0·5 to 7·0 $\mu$mole/ml., fatty acid synthesis in the control preparation showed the usual rise to a maximum followed by a decline. In the treated preparation synthesis rose towards

the control level but did not reach the level observed in the control preparation with no added ATP, even when the concentration of added ATP was raised to 7 $\mu$mole/ml. Cholesterol synthesis in the control flasks showed the usual depression at all concentrations of added ATP. In the treated preparation cholesterol synthesis rose to a maximum at 1–2 $\mu$mole ATP/ml. and then declined more or less in parallel with the control curve.

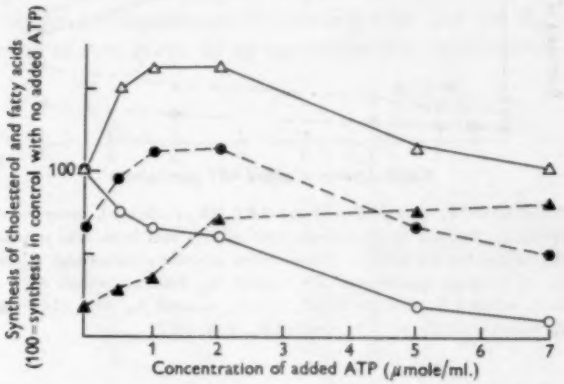



Fig. 6. Effect of ATP upon synthesis of cholesterol and fatty acids in $S_{10}$ fraction of liver from control rats and from rats injected with 30 $\mu$g L-thyroxine/day for 9 days. Values show amount synthesized expressed as percentage of amount synthesized by control $S_{10}$ with no added ATP. ○—○, cholesterol in control $S_{10}$; ●– – –●, cholesterol in treated $S_{10}$; △—△, fatty acids in control $S_{10}$; ▲– – –▲, fatty acids in treated $S_{10}$.

At the maximum value cholesterol synthesis was a little higher than the value observed in the control preparation in the standard medium alone.

After larger doses of thyroxine (50–100 $\mu$g/day) or, as sometimes happened, when there was a marked depression of lipid synthesis after 30 $\mu$g thyroxine/day, ATP had little effect on lipid synthesis until the concentration was raised above 5 $\mu$mole/ml. Figures 7 and 8 show the results of an experiment which illustrates this type of response. The treated rats were given 50 $\mu$g thyroxine/day and this was followed by a depression of fatty acid synthesis to 7 %, and of cholesterol synthesis to 36 %, of the control levels. When the concentration of added ATP was raised to 7 $\mu$mole/ml., fatty acid synthesis was still only about 20 % of the level observed in the control preparation with no added ATP. The addition of ATP at increasing concentrations to the treated preparation led to a rise in the synthesis of cholesterol, in contrast to the continuous fall observed in the control. However, synthesis failed to reach the control level when the concentration of added ATP was 7 $\mu$mole/ml.

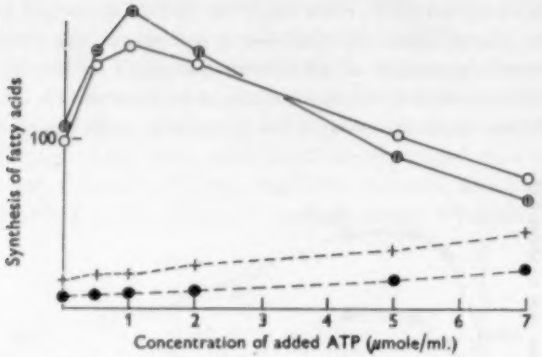



Fig. 7. Effect of ATP, with and without G6P (15 μmole/ml.), upon synthesis of fatty acids in $S_{10}$ fraction of liver from control rats and from rats injected with 50 μg L-thyroxine/day for 9 days. Values show amount synthesized, expressed as percentage of amount synthesized by control $S_{10}$ with no added ATP and no G6P. ○—○, control $S_{10}$ with no G6P; ⊕—⊕, control $S_{10}$ with G6P; ●- - -●, treated $S_{10}$ with no G6P; + - - +, treated $S_{10}$ with G6P.

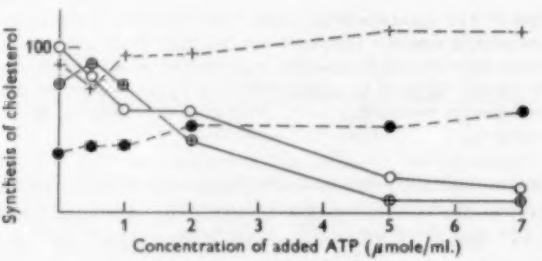



Fig. 8. Effect of ATP, with and without G6P, upon synthesis of cholesterol in $S_{10}$ fraction of liver from same rats as those used for experiment of Fig. 7. Symbols as in Fig. 7.

### *The influence of glycogen on the effect of adding ATP to $S_{10}$*

The results described in the previous section show that ATP added at a concentration of 5 μmole/ml. markedly inhibits cholesterol synthesis in the control $S_{10}$, whereas the addition of ATP at this concentration may stimulate synthesis in the treated $S_{10}$ (as in Fig. 8). Similarly, high concentrations of ATP may be inhibitory to fatty acid synthesis in the controls but stimulatory in the treated. The reason for this anomaly seems to be the deficiency of glycogen in the treated preparations.

This leads to a rapid disappearance of ATP, so that although the initial concentration may be almost as high in the treated as in the controls, the mean concentration throughout the incubation is much lower in the treated, and is not high enough to inhibit cholesterol synthesis. When glycogen is added to the treated preparation, the effect of increasing concentrations of ATP on cholesterol and fatty acid synthesis is the same as in control preparations without added glycogen. Figure 9 shows an experiment illustrating this. In the $S_{10}$ preparation from a group of rats given 50 $\mu$g thyroxine/day, synthesis of both lipids

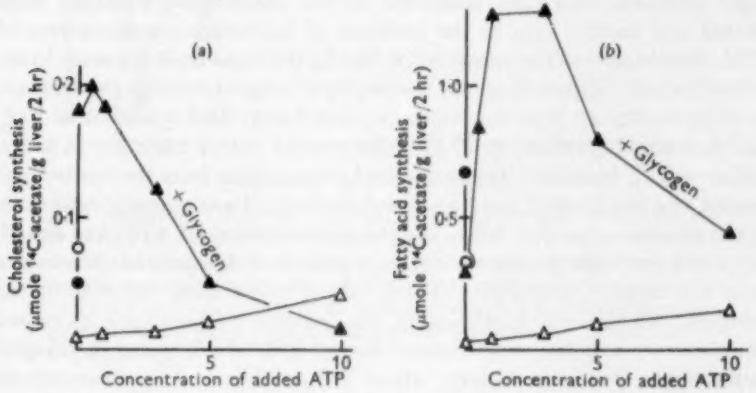



Fig. 9. Effect of ATP on cholesterol (*a*) and fatty acid (*b*) synthesis in treated $S_{10}$ after incubation at 37 °C for 2 hr with (▲—▲) and without (△—△) glycogen (30 mg/ml. added at zero time). In this experiment synthesis was also measured in control $S_{10}$, to which no ATP had been added, with (●) and without (○) glycogen (30 mg/ml.).

increased as the concentration of ATP was raised to 10 $\mu$mole/ml. When glycogen alone (30 mg/ml.) was added to the treated preparation, cholesterol synthesis was increased about twenty-fold, reaching a value more than twice that of the control preparation with no exogenous glycogen. When ATP was added at increasing concentrations to the treated preparation containing exogenous glycogen, there was a progressive fall in cholesterol synthesis. In the presence of exogenous glycogen, fatty acid synthesis in the treated preparation followed a curve similar to that seen in control preparations, rising to a maximum at 3 $\mu$mole ATP/ml. and then declining.

### *Optimal concentration of ATP for lipid synthesis in $S_{10}$*

The inhibitory action of exogenous ATP on cholesterol synthesis in the control $S_{10}$ (Fig. 5) suggests that the concentration which is optimal for synthesis may be lower than the concentration of endogenous ATP in normal

liver. We therefore tried to measure the optimal concentration of ATP by making observations on $S_{10}$ fractions from which the endogenous ATP had been removed by dialysis. $S_{104}$ and *M* fractions were prepared from the pooled livers of normal rats and from rats injected with 30 $\mu$g thyroxine/day. The microsomes in the *M* fraction were separated from the glycogen and stored at $-15°$ C until required. The $S_{104}$ was then dialysed for 18 hr at 0° C against the buffer used for preparing the homogenates. After dialysis the treated and control $S_{104}$ fractions were recombined with their own microsomes and supplemented with the substances shown in Fig. 10. Lipid synthesis was then measured in the recombined fractions from treated and control rats, in the presence of increasing concentrations of ATP. Synthesis was also measured in the $S_{10}$ fractions from the same livers before dialysis. Figure 10 shows the results of a representative experiment, in which treatment with thyroxine depressed fatty acid synthesis to 19% and cholesterol synthesis to 37% of the control values when tested in the undialysed $S_{10}$ fractions. In the dialysed preparations from the treated and control rats synthesis of fatty acids and cholesterol was scarcely detectable in the absence of added ATP. As the concentration of ATP was raised, fatty acid synthesis increased to a maximum at 2–4 $\mu$mole/ml., the values from the treated preparation being rather lower than those from the controls. Cholesterol synthesis in the control preparations increased steeply to a maximum at a concentration of ATP which varied in different experiments, but was usually about 0·5 $\mu$mole/ml. At concentrations greater than 1 $\mu$mole/ml., ATP increasingly depressed cholesterol synthesis. In the treated preparations the maximal synthesis of cholesterol was always higher than that in the controls and the inhibitory effect of ATP at high concentrations was less marked.

*Effect of changing the ATP:$Mg^{2+}$ ratio*

Some of the effects of thyroxine on mammalian cells can be reversed by the addition of Mg *in vitro* (Bain, 1954; Tapley, 1956). Although Mg was added to the buffer used in our work, it seemed desirable to find out whether an increase in the concentration of magnesium had any effect on the synthesis of lipids in the $S_{10}$ fractions from treated rats. Another reason for studying the effect of $Mg^{2+}$ was the finding of Lévy & Popják (1960) that there is an optimal ATP: $Mg^{2+}$ ratio for one of the steps in the synthesis of cholesterol.

When no ATP was added, increasing the Mg concentration from 5 to 10 $\mu$mole/ml. depressed cholesterol synthesis in the control preparations and had no effect on the treated (Fig. 11). At higher concentrations of ATP, the inhibitory effect of ATP in the control preparation was partially reversed; synthesis of cholesterol in the treated preparations was un-

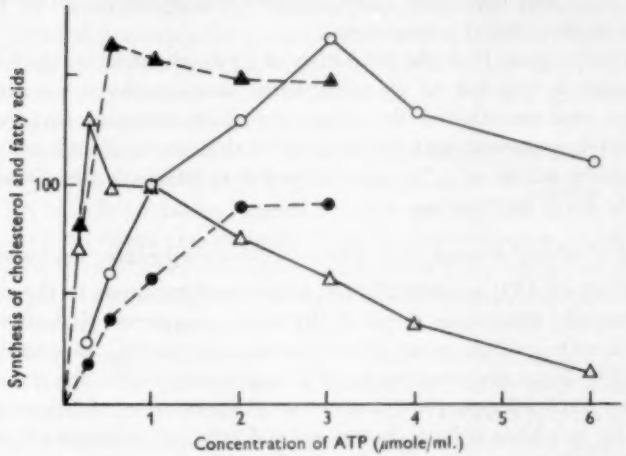



**Fig. 10.** Synthesis of cholesterol and fatty acids at increasing concentrations of ATP in dialysed $S_{10}$ from control and thyroxine-treated rats. Values expressed as percentage of synthesis in control $S_{10}$ in the presence of 1 μmole ATP/ml. ○—○, fatty acid synthesis in control; ●- - -●, fatty acid synthesis in treated; △—△, cholesterol synthesis in control; ▲- - -▲, cholesterol synthesis in treated preparation. Samples incubated at 37° C for 2 hr. in standard medium + CoA (0·05 μmole) and glycogen (35 mg).

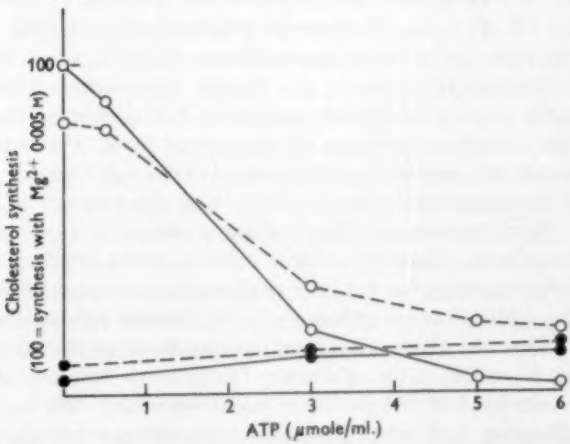



**Fig. 11.** Effect of changing the relative concentrations of $Mg^{2+}$ and ATP on synthesis of cholesterol in control (○) and treated (●) $S_{10}$ fractions incubated at 37 °C for 2 hr. Synthesis was measured in the standard medium at increasing concentrations of ATP with 0·005 M-$Mg^{2+}$ (———) or 0·01 M-$Mg^{2+}$ (– – – –). Values expressed as % synthesis in control $S_{10}$ with 0·005 M-$Mg^{2+}$ and no added ATP. The treated $S_{10}$ fraction was obtained from a group of rats given 50 μg thyroxine/day for 9 days.

affected. $Mg^{2+}$ had very little effect on fatty acid synthesis either in the control or in the treated preparations.

These results show that the inhibition of lipid synthesis brought about by thyroxine is not due to an antagonism between thyroxine and the magnesium ions essential for the action of certain enzymes. Our findings are, however, consistent with the possibility that some, though not all, of the inhibitory action of ATP on cholesterol synthesis is due to an unfavourable ATP: $Mg^{2+}$ ratio.

*Effect of adding generators of reduced triphosphopyridine nucleotide*

The failure of ATP consistently to restore lipid synthesis to the normal level, especially after large doses of thyroxine, suggested that thyroxine treatment may produce some other shortage in the $S_{10}$, in addition to that of ATP. Since the break-down of glycogen may lead to the generation of reduced triphosphopyridine nucleotide (TPNH), one of the cofactors required for synthesis of both cholesterol and fatty acids, it seemed possible that the low glycogen content of the $S_{10}$ might lead to a shortage of TPNH. Accordingly, experiments were designed to test the effect of substances capable of reducing TPN to TPNH in the $S_{10}$ fraction from rats treated with 50 $\mu$g thyroxine/day.

*Effect of glucose-6-phosphate.* When glucose-6-phosphate (G6P, 15 $\mu$mole/ml.) was added to the control preparations, fatty acid synthesis was either uninfluenced, or was slightly stimulated in the presence of low concentrations of ATP (Fig. 7). Cholesterol synthesis showed little change, except in the presence of high concentrations of ATP, when inhibition was always observed (Fig. 8). In the treated preparations G6P stimulated fatty acid synthesis at all concentrations of ATP tested (Fig. 7), but in no case did it restore synthesis to the normal level. Cholesterol synthesis in the treated preparations was stimulated at all concentrations of ATP and in one experiment (shown in Fig. 8) it was restored to the level observed in the control preparation without added ATP.

*Effect of iso-citrate.* Since the soluble fraction of the liver cell contains *iso*-citrate dehydrogenase (an enzyme catalysing the oxidation of *iso*-citrate by TPN), the addition of *iso*-citrate to the $S_{10}$ should increase the supply of TPNH. However, the oxidation of *iso*-citrate gives rise to $CO_2$, and this is known to stimulate the synthesis of fatty acids from acetate by the malonyl co-enzyme-A (CoA) pathway (see Discussion). We have shown elsewhere (Fletcher & Myant, 1961) that fatty acids in our $S_{10}$ preparations are synthesized by this pathway. It was therefore necessary to work with a concentration of $HCO_3^-$ optimal for fatty acid synthesis if we were to distinguish between a stimulatory action of *iso*-citrate on fatty acid synthesis due to TPNH generation, and one due to an increase

in $HCO_3^-$ concentration. Preliminary experiments showed that fatty acid synthesis increased to a maximum as the concentration of $HCO_3^-$ was raised to 30 $\mu$mole/ml. and that inhibition occurred at higher concentrations. A concentration of 30 $\mu$mole/ml. $HCO_3^-$ was therefore chosen for the experiments with *iso*-citrate.

Figure 12(*a*, *b*) shows the results of an experiment in which the treated $S_{10}$ fraction was obtained from a group of rats given 50 $\mu$g thyroxine/day. Synthesis of both cholesterol and fatty acids was reduced to less than 10% of that in the control preparation. When the concentration of added ATP was raised from 0 to 10 $\mu$mole/ml. synthesis of fatty acids increased progressively in the treated preparation, though it did not reach the maximum value observed in the controls. When DL-*iso*-citrate (6 $\mu$mole/ml.) was added, fatty acid synthesis at a given concentration of ATP was unchanged in the control, but was stimulated in the treated preparation; with *iso*-citrate in the presence of 10 $\mu$mole ATP/ml., synthesis of fatty acids in the treated preparation reached a higher level than that observed in the control.

Cholesterol synthesis in the treated preparation was restored almost to the control level by the addition of 10 $\mu$mole ATP/ml. At all concentrations of ATP, *iso*-citrate depressed cholesterol synthesis in both treated and control preparations.

### *Effect of TPN, DPNH and co-enzyme A*

When the concentration of TPN was increased above that already present in the standard medium, synthesis of fatty acids and cholesterol was reduced, both in the control and treated preparations, and in the presence of increasing concentrations of ATP (Fig. 13). When the concentration of DPNH was increased in the standard medium with 3 $\mu$mole ATP/ml., fatty acid and cholesterol synthesis was depressed in both treated and control $S_{10}$ preparations (Table 1). The addition of co-enzyme A (0·05 $\mu$mole/ml.) to the standard medium supplemented with ATP had no effect on fatty acid synthesis, either in the control or in the treated $S_{10}$, but cholesterol synthesis was stimulated to an equal extent in both preparations (Table 1). CoA at concentrations greater than 0·1 $\mu$mole/ml. depressed cholesterol synthesis in both preparations.

### *Relation between fatty acid and cholesterol synthesis*

The results reported here and in our two earlier papers (Fletcher & Myant, 1958, 1960) show that in certain conditions the synthesis of cholesterol is related reciprocally to that of fatty acids. In the $S_{10}$ from normal rats, for instance, fatty acid synthesis is stimulated by increasing the concentration of ATP to 4 $\mu$mole/ml., whereas cholesterol synthesis

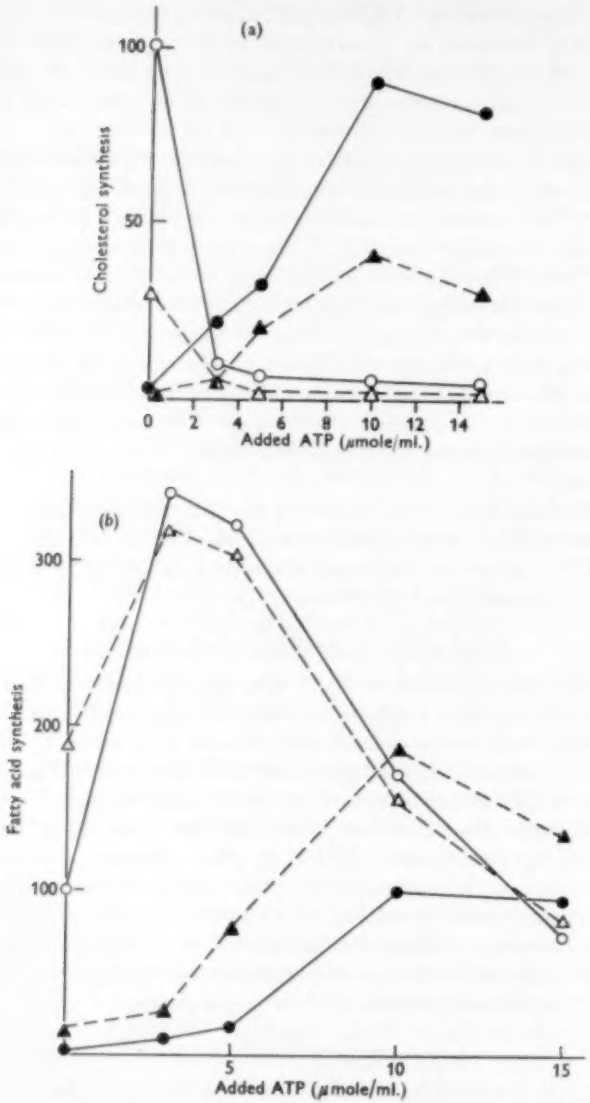



Fig. 12. Effect of DL-*iso*-citrate (20 μmole/ml.), in the presence of varying concentrations of ATP, on cholesterol (*a*) and fatty acid (*b*) synthesis in $S_{10}$ from control rats and from rats injected with 50 μg thyroxine/day for 9 days. Samples incubated in standard medium with 30 μmole $HCO_3^-$/ml. at 37° C for 2 hr. Values expressed as % synthesis in control $S_{10}$ with no *iso*-citrate and no added ATP. ○—○, control with no *iso*-citrate; ●—● treated with no *iso*-citrate; △---△, control with *iso*-citrate; ▲---▲, treated with *iso*-citrate.

is inhibited. Similarly, in liver slices from thyroxine-treated rats and in the $S_{10}$ from rats given small doses of thyroxine, cholesterol synthesis is increased and fatty acid synthesis is depressed. This relationship suggests that the two biosyntheses are interdependent. We have shown elsewhere (Fletcher & Myant, 1961) that synthesis of fatty acids in the $S_{10}$ can be modified by the addition of avidin without influencing cholesterol synthesis. Nevertheless, it seemed worth while examining this point in more detail. The effect of increasing concentrations of ATP on cholesterol synthesis in the $S_{10}$ was tested in the presence of enough avidin to inhibit fatty acid synthesis to about 20% of the normal level. At the lower

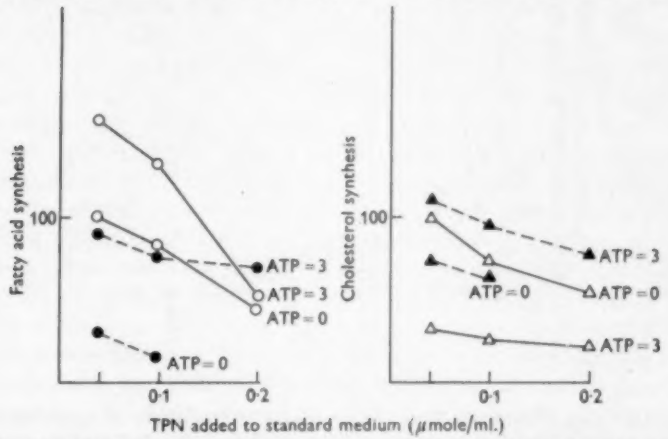



**Fig. 13.** Effect of TPN on synthesis of fatty acids (○, ●) and cholesterol (△, ▲) in control (○, △) and treated (●, ▲) $S_{10}$ fractions with no added ATP and with 3 μmole added ATP/ml. Treated rats given 30 μg L-thyroxine/day for 9 days. Samples incubated at 37° C for 2 hr in standard medium supplemented with TPN at the concentrations shown. Values expressed as % synthesis in control $S_{10}$ in standard medium with 0·05 μmole TPN/ml. ATP concentrations as shown in Fig.

**Table 1.** Effect of DPNH and CoA on fatty acid and cholesterol synthesis in control and treated $S_{10}$ samples incubated for 2 hr in standard medium with 3 μmole ATP/ml., with and without DPNH (0·1 μmole/ml. in addition to that already present) or CoA (0·05 μmole/ml.). Values show average μmole 1-$^{14}$C-acetate incorporated/g liver during incubation of paired samples. Treated rats given 30 μg thyroxine/day

| | | Synthesis of lipids in Standard medium | | |
|---|---|---|---|---|
| | | + ATP | + ATP + DPNH | + ATP + CoA |
| Fatty acids | Control | 0·213 | 0·174 | 0·204 |
| | Treated | 0·026 | 0·022 | 0·027 |
| Cholesterol (× 10) | Control | 0·420 | 0·271 | 0·497 |
| | Treated | 0·072 | 0·054 | 0·088 |

concentrations of ATP, inhibition of fatty acid synthesis led to a slight increase in cholesterol synthesis (Fig. 14), but the inhibitory action of ATP at high concentrations was just as marked when fatty acid synthesis was inhibited as it was in the absence of avidin.

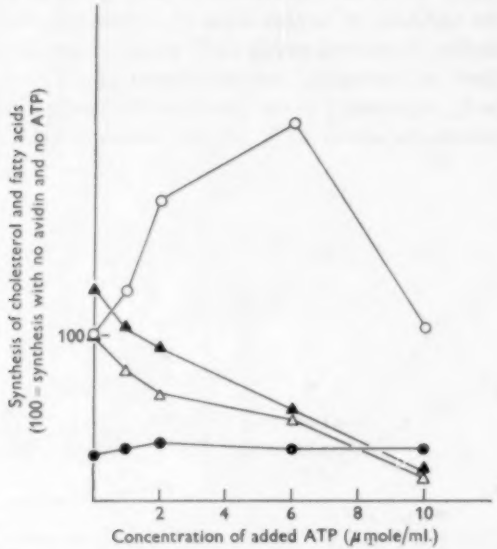



Fig. 14. Effect of avidin on fatty acid (○, ●) and cholesterol (△, ▲) synthesis in $S_{10}$ from normal rat liver incubated at 37 °C for 2 hr in standard medium with ATP at increasing concentrations. Avidin added equivalent to 2 μg biotin/3·5 ml. incubation mixture. ○, △, no avidin; ●, ▲, with avidin. Values expressed as % synthesis with no added ATP and no avidin.

## DISCUSSION

### *Depression of lipid synthesis in $S_{10}$*

Toxic doses of thyroid hormone have been known for many years to cause a fall in the glycogen content of the liver (Parhon, 1913). Our observations show that there is a significant fall in liver glycogen after the smallest doses capable of depressing lipid synthesis in the $S_{10}$ fraction. This is consistent with our conclusion (Fletcher & Myant, 1960) that a shortage of glycogen is the most important cause of the depression of lipid synthesis in the $S_{10}$. The experiments on the effect of adding glycogen to the treated $S_{10}$ show that glycogen normally supplies energy for the resynthesis of ATP (Fig. 4). This, together with the finding that ATP stimulates lipid synthesis in the treated preparations, also supports our

suggestion that the fall in glycogen acts, at least in part, by producing a shortage of ATP during the incubation. The diminution in the concentration of pre-formed ATP in the $S_{10}$ may also contribute to the depression of lipid synthesis. However, the amount of lipid synthesized during the 2-hr period probably depends more upon ATP regenerated throughout the incubation than upon the amount initially present, since ATP disappears rapidly unless glycogen is present (Fig. 3).

A lack of ATP could, by itself, account for the depression of cholesterol synthesis observed after doses of 30 $\mu$g of thyroxine, since ATP usually restored synthesis to the normal level. The failure to restore fatty acid synthesis completely with ATP alone, especially after the larger doses of thyroxine, suggests that the depression of this synthesis is due to a deficiency of other cofactors, in addition to that of ATP. The results of Table 1 show that there is no shortage of DPNH or CoA in the treated $S_{10}$, but the stimulatory effect of *iso*-citrate points to a failure to supply enough TPNH for fatty acid synthesis. This, in turn, could be due to the low glycogen content of the $S_{10}$, since TPNH is generated in the cell sap partly by the oxidation of G6P, which may arise from the break-down of glycogen. The stimulatory effect of G6P is also consistent with a shortage of TPNH, although it is impossible to tell whether this effect is due to the formation of TPNH, of ATP, or of both, since G6P generates ATP as well as TPNH. Glock & McLean (1955) have shown that large doses of thyroxine reduce the concentration of total triphosphopyridine nucleotide (TPN + TPNH) in whole liver. However, this may not be relevant to what happens in the $S_{10}$ fraction after daily doses of 30–50 $\mu$g thyroxine; the inhibitory effect of TPN (Fig. 13) shows that there is no shortage of this cofactor in our preparations.

Although a shortage of TPNH may be one of the factors causing a depression of fatty acid synthesis in the treated $S_{10}$, it does not seem to contribute to the depression of cholesterol synthesis, since this was never stimulated by *iso*-citrate. Presumably the enzyme systems that synthesize cholesterol are less sensitive to a fall in TPNH than are those taking part in fatty acid synthesis.

Fatty acid synthesis could never be restored to the maximum in the controls by adding ATP and *iso*-citrate in excess. The depression in the treated $S_{10}$ cannot, therefore, be fully explained by a shortage of ATP and TPNH. Thyroxine must have some other effect, in addition to that of causing a shortage of the cofactors generated by glycogen in the control $S_{10}$ fraction, but we do not know what this is. Possibly, treatment with thyroxine diminishes the activity of some of the enzymes that synthesize fatty acids in the liver. The observations on dialysed $S_{10}$ preparations are consistent with this, in that fatty acid synthesis was usually less in

the treated than in the control preparation when both were dialysed and tested in the standard medium supplemented with the same substances (CoA, ATP and glycogen).

*Effect of thyroxine on lipid synthesis in liver slices*

Treatment with thyroxine brings about a fall in the ATP concentration in the whole liver, detectable after the smallest doses capable of influencing lipid synthesis (Fig. 2). Other workers (Chilson & Sacks, 1959; Chatagner & Gautheron, 1960) have observed this effect of thyroxine, though not with doses as small as those we have used. Since ATP in the whole cell is normally regenerated in the mitochondria, the fall in ATP concentration cannot be explained by the low glycogen content of the liver in thyroxine-treated rats. Thyroxine added *in vitro* is known to inhibit oxidative phosphorylation in mitochondria, either directly (Hoch & Lipmann, 1954) or indirectly by accelerating mitochondrial swelling (Tapley, 1956). However, the evidence that thyroxine affects oxidative phosphorylation *in vivo* is based on experiments in which animals were treated with doses of thyroxine many times larger than those used in this work (Tapley, 1956; Lee, Takemori & Lardy, 1959). Moreover, observations on the liver mitochondria of our control and treated rats (K. Fletcher, N. B. Myant, and D. D. Tyler, unpublished) have shown either no differences in the efficiency of oxidative phosphorylation, or differences that seem to be too small to account for the observed fall in the ATP content of the whole liver. It has been claimed that thyroxine stimulates a mitochondrial ATP-ase (Maley, 1957), but if this were the cause of the fall in ATP in the livers of the treated rats a decrease in the efficiency of oxidative phosphorylation should be readily detectable in the mitochondria. Possibly, high-energy phosphate is generated normally in the liver mitochondria of the treated rats and is destroyed abnormally rapidly in the cell sap. However, this is difficult to reconcile with our finding that the rate of disappearance of ATP is normal in treated $S_{10}$ preparations enriched with glycogen (Fig. 4).

The fall in ATP in the whole cell, however it may be brought about, would be expected to lead to a depression of fatty acid synthesis in slices from treated livers. The findings in the $S_{10}$ preparation suggest that there may also be a shortage of TPNH owing to the low glycogen content of the liver cells, and that there may be a reduction in the activity of the fatty-acid-synthesizing enzymes. Thus, our findings provide a reasonable explanation of the effect of thyroxine treatment on fatty acid synthesis in slices. However, cholesterol synthesis from acetate is always stimulated in slices from thyroxine-treated rats (Fletcher & Myant, 1958). This raises the question whether a stimulation in the whole cell can be

explained by the effects that we have observed in the $S_{10}$ fraction. In this connexion it is relevant to consider how far the syntheses of fatty acids and cholesterol are interdependent, both in the $S_{10}$ fraction of the liver and in the whole cell.

It has been shown (Fletcher & Myant, 1961) that in the $S_{10}$ fractions used in this work fatty acids are synthesized by the 'malonate' pathway discovered by Brady (1958) and Wakil (1958). Acetate, after activation to acetyl CoA, is converted to malonyl CoA by the addition of $CO_2$. The fatty acid molecule is then built up by the condensation of units of malonyl CoA. In addition to ATP and CoA, TPNH and possibly DPNH are required as cofactors. The formation of acetyl CoA is also the first step in the synthesis of cholesterol, but the next step is the formation of aceto-acetyl CoA and not of malonyl CoA. ATP, CoA and TPNH are also required for this synthesis. Thus, the two biosynthetic pathways share a single precursor (acetate) and at least three cofactors. A change in the amount of lipid synthesized by one pathway might, therefore, be expected to influence the amount synthesized by the other. It is unlikely that competition for acetate influences the relative rates of the two syntheses in the $S_{10}$, since it was added in excess. There could, however, be competition for some cofactor. Let us suppose that fatty acid synthesis is limited by the supply of ATP and that cholesterol synthesis is limited by the amount of some other cofactor ($x$) common to the two pathways. An increase in ATP would stimulate fatty acid synthesis and this would lead to a fall in the amount of $x$ available, and, hence, to a depression of cholesterol synthesis (as in Fig. 5). Similarly, a fall in ATP would depress fatty acid synthesis and so release more of $x$. Provided the concentration of ATP were not so low as to limit cholesterol synthesis, this would increase. At lower concentrations of ATP both fatty acid and cholesterol synthesis would be depressed.

This hypothesis depends on the assumption that fatty acid and cholesterol synthesis are, in fact, interdependent. Against this, we have shown that in the $S_{10}$ fraction it is possible to produce large changes in fatty acid synthesis without changing cholesterol synthesis (Fletcher & Myant, 1961). This suggests that, in the conditions in which synthesis is observed in the $S_{10}$, the two pathways are not closely linked by competition for cofactors. The finding that ATP still inhibits cholesterol synthesis when fatty acid synthesis is suppressed by avidin (Fig. 14) is also inconsistent with the explanation we have suggested above for the action of ATP on cholesterol synthesis in the control $S_{10}$ fractions. However, the concentrations of endogenous cofactors in the whole cell probably differ widely from the concentrations of added cofactors in the $S_{10}$ fraction. It is possible, therefore, that conditions in liver slices are such as to favour com-

petition of the type discussed above. In this case, a fall in the concentration of ATP might lead to an increase in cholesterol synthesis. It is, perhaps, relevant that Bloomfield & Bloch (1960) have shown that sterol synthesis from acetate is increased in a biotin-requiring strain of yeast when fatty acid synthesis is reduced by withholding biotin from the incubation medium.

Another factor which may help to explain how a shortage of ATP could cause a rise in cholesterol synthesis is suggested by the ATP concentration curves from dialysed preparations (Fig. 10). These curves show that the optimal concentration of ATP is about 0·5 $\mu$mole/ml. for cholesterol synthesis and is about 3 $\mu$mole/ml. for fatty acid synthesis. A similar optimal concentration of ATP for cholesterol synthesis was also found by Knauss, Porter & Wasson (1959). The concentration of ATP in the livers of the untreated rats used in this work was 2·4 $\mu$mole/g wet liver (Fig. 2). If, therefore, the relationship between ATP concentration and lipid synthesis is the same in the whole cell as it is in the $S_{10}$, a fall in liver ATP comparable with that shown in Fig. 2 would be expected to lead to an increase in cholesterol synthesis in liver slices.

Finally, in the dialysed preparations synthesis of cholesterol was always greater in the treated than in the control preparations (Fig. 10). Furthermore, when glycogen was added to the treated $S_{10}$, cholesterol synthesis was usually 2–3 times as great as that in the controls (Fig. 9). These observations suggest that the activity of the cholesterol-synthesizing enzymes per unit weight of tissue is increased in the treated preparations.

## SUMMARY

1. When rats are given doses of thyroxine increasing from 10 to 50 $\mu$g/day, there is a progressive fall in the concentration of glycogen and adenosine triphosphate (ATP) in the liver. The fall in glycogen is associated with a depression of synthesis of fatty acids and cholesterol in the $S_{10}$ fraction.

2. The depression of cholesterol synthesis can usually be reversed by ATP; that of fatty acids can be partially reversed by ATP plus a generator of reduced triphosphopyridine nucleotide (TPNH).

3. In $S_{10}$ fractions from treated rats endogenous and exogenous ATP disappears rapidly during incubation unless glycogen is added initially. If glycogen is added after the ATP has disappeared, ATP reappears. It is concluded that glycogen is the main source of energy for the regeneration of ATP in the $S_{10}$ and that the fall in glycogen leads to a shortage of the ATP necessary for cholesterol and fatty acid synthesis. A moderate shortage of TPNH, sufficient to limit the synthesis of fatty acids but not of cholesterol, may also result from the fall in glycogen.

4. The optimal concentration of ATP for cholesterol synthesis in dialysed $S_{10}$ preparations is less than the concentration of endogenous ATP in normal liver. For fatty acid synthesis the optimal concentration is greater than the concentration in normal liver.

5. It is pointed out that the fall in the ATP content of whole liver after thyroxine treatment might explain why cholesterol synthesis is increased, and why fatty acid synthesis is depressed, in slices from the livers of thyroxine-treated rats (Fletcher & Myant, 1958).

6. The possibility that fatty acid and cholesterol synthesis are interdependent in the whole cell is also discussed. It is shown that this could lead to an increase in cholesterol synthesis when fatty acid synthesis is limited by a shortage of ATP.

## REFERENCES


Bain, J. A. (1954). The effect of magnesium upon thyroxine inhibition of phosphorylation. *J. Pharmacol.* **110**, 2–3.

Bloomfield, D. K. & Bloch, K. (1960). The formation of $\Delta^9$-unsaturated fatty acids. *J. biol. Chem.* **235**, 337–345.

Brady, R. O. (1958). The enzymatic synthesis of fatty acids by aldol condensation. *Proc. nat. Acad. Sci., Wash.*, **44**, 993–998.

Bucher, N. L. R. & McGarrahan, K. (1956). The biosynthesis of cholesterol from acetate 1-$C^{14}$ by cellular fractions of rat liver. *J. biol. Chem.* **222**, 1–15.

Chatagner, F. & Gautheron, D. (1960). Influence des hormones thyroïdiennes sur la teneur en adenosine triphosphate du foie du rat. *Biochim. biophys. acta*, **41**, 544–545.

Chilson, O. P. & Sacks, J. (1959). Effect of hyperthyroidism on distribution of adenosine phosphates and glycogen in liver. *Proc. Soc. exp. Biol., N.Y.*, **101**, 331–335.

Fletcher, K. & Myant, N. B. (1958). Influence of the thyroid on the synthesis of cholesterol by liver and skin *in vitro*. *J. Physiol.* **144**, 361–372.

Fletcher, K. & Myant, N. B. (1960). Effects of thyroxine on the synthesis of cholesterol and fatty acids by cell-free fractions of rat liver. *J. Physiol.* **154**, 145–152.

Fletcher, K. & Myant, N. B. (1961). The effect of some cofactors on the synthesis of fatty acids and cholesterol in cell-free preparations of rat liver. *J. Physiol.* **155**, 498–505.

Glock, G. E. & McLean, P. (1955). Effects of hormones on levels of oxidized and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide in liver and diaphragm. *Biochem. J.* **61**, 397–402.

Hoch, F. L. & Lipmann, F. (1954). The uncoupling of respiration and phosphorylation by thyroid hormones. *Proc. nat. Acad. Sci., Wash.*, **40**, 909–921.

Knauss, H. J., Porter, J. W. & Wasson, G. (1959). The biosynthesis of mevalonic acid from 1-C14-acetate by a rat liver enzyme system. *J. biol. Chem.* **234**, 2835–2840.

Lee, Y.-P., Takemori, A. E. & Lardy, H. (1959). Enhanced oxidation of α-glycerophosphate by mitochondria of thyroid-fed rats. *J. biol. Chem.* **234**, 3051–3054.

Levy, H. R. & Popják, G. (1960). Studies on the biosynthesis of cholesterol. 10. Mevalonic kinase and phosphomevalonic kinase from liver. *Biochem. J.* **75**, 417–428.

Maley, G. F. (1957). Comparison of some enzyme systems in normal and thyrotoxic rat livers. *Amer. J. Physiol.* **188**, 35–39.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

Parhon, M. (1913). Sur la teneur en glycogène du foie et des muscles chez les animaux traités par des préparations thyroïdiennes. *J. Physiol. Path. gén.* **15**, 75–78.

Somogyi, M. (1945). A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 160, 61–68

SOMOGYI, M. (1957). Preparation of glycogen, nitrogen- and phosphorus-free, from liver. In *Methods in Enzymology*, ed. COLOWICK, S. P. & KAPLAN, N. O. Vol. III, pp. 3–4. New York: Academic Press.

STREHLER, B. L. & TOTTER, J. R. (1954). Determination of ATP and related compounds: firefly luminescence and other methods. In *Methods of Biochemical Analysis*, ed. GLICK, D. Vol. I, pp. 341–356. New York: Interscience Publishers.

TAPLEY, D. F. (1956). The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. *J. biol. Chem.* **222**, 325–339.

WAKIL, S. J. (1958). A malonic acid derivative as an intermediate in fatty acid synthesis. *J. Amer. chem. Soc.* **80**, 6465.

WOOLLEY, D. W. & LONGSWORTH, L. G. (1942). Isolation of an antibiotin factor from egg white. *J. biol. Chem.* **142**, 285–290.

*J. Physiol.* (1961), **157**, *pp.* 565–580
*With 6 text-figures*
*Printed in Great Britain*



# SOME FACTORS CONCERNED IN DIFFERENTIAL NERVE BLOCK BY LOCAL ANAESTHETICS

BY P. W. NATHAN AND T. A. SEARS

*From the Medical Research Council Neurological Research Unit, and the Institute of Neurology, National Hospital for Nervous Diseases, Queen Square, London, W.C.* 1

(*Received* 6 *February* 1961)

The differential blocking of nerve fibres of different sizes by local anaesthetics was first investigated by Gasser & Erlanger (1929). They found that within the A fibre group of nerves of the frog and the dog, cocaine blocks fibres of smaller diameter and slower conduction rate before larger fibres; but as they found that 'blocking of some of the fast fibres occurs before the slow ones', they were unable to formulate a satisfactory theory to account for all the facts they observed.

The subject was re-investigated by Heinbecker, Bishop & O'Leary (1934) using procaine; and they also found that 'blocking is not complete in the slower somatic group before definite depression, from 30 to 50%, is also present in the faster groups'. They concluded 'that the order of depression of the fibres is in general in the inverse order of the diameter of the fibres, but that other factors may be involved, such as the character of the axon and the thickness of the myelin sheath'. Leksell (1945) found that ethocaine (procaine) has a greater effect on the gamma fibres than on the alpha fibres of the anterior roots, and it was later found by Matthews and Rushworth (1957) that procaine blocks gamma fibres before alpha fibres. On the other hand, Everett & Goodsell (1952) stated that C fibres or 'small slow nerve fibres' are not more sensitive to procaine than 'the large fast A fibres', and Everett & Toman (1954) found that 'A and C groups' were often blocked at the same time.

Apart from considerations of fibre size and of the presence or absence of myelination, it has often been stated that sensory nerve fibres are more susceptible to the action of local anaesthetics than motor fibres. MacIntosh (1957), for instance, states: 'It is well known that in local and spinal analgesia, sensory nerve fibres are affected before motor.' This view seems to be based on early observations, such as those of Dixon (1905), that cocaine applied to a nerve supplying a muscle blocks reflex contractions before it blocks contractions due to electrical stimulation of the nerve. Recent work by Matthews & Rushworth (1957), however, has provided an

alternative explanation; for it now seems probable that the disappearance of the reflex effects is due to selective blocking of the small motor (gamma) fibres, while the contraction due to nerve stimulation remains as long as the alpha fibres remain unblocked.

The present paper reports observations on the effects of local anaesthetics on conduction in fibres of the spinal roots; on the order of block within the A group of fibres; and on the order of block of the non-myelinated fibres compared to the myelinated. Further, it elucidates some of the factors giving rise to differential blocking; and it proposes an explanation of the inconsistency concerning blocking of fibres of different sizes.

## METHODS

The methods are essentially the same as those given in a previous paper (Nathan & Sears, 1960*a*). The experiments were performed on a total of 22 cats, anaesthetized with Nembutal (pentobarbitone, Abbott Laboratories; 35–40 mg/kg) given by intraperitoneal injection. In all the experiments the local anaesthetics were applied to the nerve roots. The lumbosacral roots and various nerves of the hind limb and tail were made available for stimulation or for recording. For studying the effects of the anaesthetics on the A fibres of the posterior roots the stimulating electrodes were applied to the roots and the recording was made from the peripheral nerve; as the peripheral nerves were divided distally, this recording of antidromic conduction produced records free from spontaneous afferent impulses without the need of widespread de-afferentation. In experiments on anterior roots both orthodromic and antidromic conduction were used. For studying the effects of anaesthetics on the C fibres, the limb-nerve preparations were unsuitable, owing to the excessive temporal dispersion of the impulses over the conduction distances encountered. It was found that the roots innervating the tail formed a suitable preparation. The laminectomy was continued to the proximal vertebrae of the tail, which exposed suitable lengths of roots ensheathed by dura mater. The roots were divided distally close to their intervertebral foramina and were placed on stimulating electrodes; the corresponding posterior roots were cut close to the spinal cord, passed through the chamber (described below), and placed on recording electrodes. In this preparation the conduction distance is shorter than in the limb-nerve spinal root preparation, and as stimulation and recording is from the same root, divided at each end, the recording is free from spontaneous afferent impulses from the periphery.

The stimulating and recording methods were the same as those used previously, except that square-wave voltage pulses were used for stimulating the C fibres, whereas previously condenser discharges had been used. The stimulator output was fed through a four-position selector switch to one of four separate potentiometers. At the beginning of an experiment each potentiometer could be adjusted to provide a stimulus, the intensity of which was adequate to excite only large fibres or both large and small fibres, according to the requirements of the experiment. It was thus possible during an experiment to select quickly a pre-set stimulus intensity which was known only to excite fibres of a given conduction rate; this procedure allowed a comparison to be made between the selective activation of the fibre group by electrical stimulation and the degree of differential blocking caused by the local anaesthetic. In all experiments the stimulus repetition interval was 1 sec.

In designing a chamber for the application of the solutions the ability of the impulse to 'hurdle' two or three inactive nodes was taken into account. According to Visozo & Young (1948) the longest internode of mammalian fibres is 1·5 mm. The internal diameter of the chamber used was 8 mm; and therefore it contained at least 5 nodes of the largest fibres. The chamber, made of Perspex, is illustrated in Fig. 1. Its cubic capacity is 1 ml. The

filament of nerve root was passed through the holes on either side of the chamber; this was carried out by tying a thread of fine silk to the cut end of the filament, threading the silk through a fine needle, and passing the needle through the holes in the chamber. The chamber was then placed deep in the trough formed by the laminectomy, in such a position that the hole on one side of the chamber was close to the region where the root passes through the dura mater. The silk was then gently pulled so as to draw as great a length as possible of the root through the chamber, without causing it to be kinked or pressed on; low-power magnification was used to control this procedure. The circular channel of the chamber was then packed with soft wax, which was firmly pressed round the nerve root, so as to make the

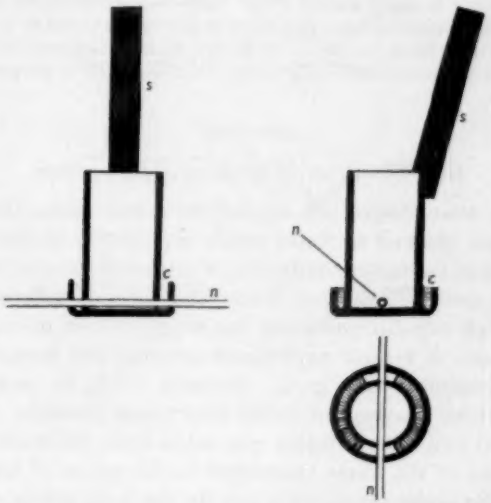



Fig. 1. Elevation and plan views of Perspex chambers; *c*, channel—shaded part is the part filled with Perspex cement; *n*, filament of nerve root passing through chamber; *s*, supporting rod.

junction between the hole and the root watertight. The wax was made from a mixture of paraffin wax (m.p. 56° C) and white soft paraffin, melted, mixed together, and allowed to cool. The proximal end of the root was placed on platinum electrodes supported near the chamber. If sampling then showed that the preparation was satisfactory for displaying both the fast-conducting and slowly-conducting fibres (which is not always the case), the entire trough formed by the laminectomy was covered by paraffin oil equilibrated with oxygen and carbon dioxide. The Perspex chamber which projected above the level of the paraffin oil was filled with Ringer–Locke solution.

The local anaesthetics used were cocaine, procaine and lignocaine. They were dissolved either in Ringer–Locke solution or in a modified Krebs–Henseleit solution. The Ringer–Locke solution had the following composition (mM): NaCl, 145; KCl, 5·7; $CaCl_2$, 2·2; $NaHCO_3$, 2·1; glucose, 5·5. The Krebs–Henseleit solution was closely similar to that modified by Diamond, Gray & Inman (1958), and had the following composition (mM): NaCl, 145; $CaCl_2$, 2·5; $MgSO_4$, 1·2; $KH_2PO_4$, 0·58; $K_2HPO_4$, 2·5; glucose, 5·5. This solution differed from that of Diamond *et al.* in that no HCl was added to it. The reason for using this solution was that some of the experiments reported here were done in conjunction with experiments

on the differential blocking induced by sodium-deficient solutions (Nathan & Sears, 1960*b*). The pH of the solutions was tested on a pH meter and was found to lie between 7 and 7·4. Before use they were equilibrated with 5 % $CO_2$ and 95 % $O_2$.

The nomenclature used here for labelling the fibre groups combines various current usages. Thus, following Erlanger & Gasser (1937), the A fibres conducting at 80–120 m/sec are called alpha fibres, those conducting from 60 to 80 m/sec are called beta, those conducting from 40 to 60 m/sec are called gamma, and those conducting at rates of less than 40 m/sec are called delta. The term 'fast-conducting' applied to the nerve fibres of the lumbosacral roots refers to the alpha and beta fibres, such as those supplying the extrafusal muscle fibres. The small fibres of the anterior root are referred to in the literature as either the small-fibre groups or as the gamma group. Here they will be called the gamma-delta fibres, because their conduction rates were found to be between 15 and 50 m/sec, with a peak at 30 m/sec. This same range was found by Kuffler, Hunt & Quilliam (1951). In the tail-nerve preparation the fastest conducting fibres were found to be in the gamma range.

## RESULTS

### *Differential rate of blocking of fibre groups*

When local anaesthetics are applied to spinal roots, the constituent nerve fibres are blocked at times which are related to their conduction rates; yet some of the faster-conducting fibres are blocked at the same time as some more slowly-conducting fibres. This form of differential blocking was found in all experiments when the concentration of anaesthetic was 0·1 % or higher. A typical experiment showing this form of differential blocking is illustrated in Fig. 2. Procaine 0·1 % in modified Krebs–Henseleit solution was applied to the first sacral posterior root; the root was stimulated and the recording was made from the sciatic nerve. The conduction rate of the fibres (measured to the peaks of the waves) was 80 m/sec for the alpha group, 65 m/sec for the beta group, and 12–19 m/sec for the delta group. The records on the left display the action potentials of the fast-conducting fibres, and those on the right display the action potentials of the slowly-conducting fibres. The latter consist of 10 superimposed sweeps: 5 sweeps were recorded at a stimulus intensity which, before the application of the procaine, had been adjusted to be below threshold for the delta group; the other 5 sweeps were recorded at a stimulus intensity giving a maximal response of this group of fibres.

Within 45 sec of filling the chamber with 0·1 % procaine in modified Krebs–Henseleit solution the wave of the delta group was considerably reduced. The height of the alpha spike at this time was diminished by 4 %; the beta wave, which in the control record appears as the notch in the descending phase of the alpha spike, was diminished by 20 % of the original height of the notch on the alpha spike. By 2 min 15 sec the delta wave had gone, to the extent that the record was barely distinguishable from that obtained with the stimulus intensity set below threshold for these small fibres; at this time the alpha spike was reduced by 8 %. The

fall-out of the delta wave can also be seen in the records taken at low amplification. At 4 min 45 sec the alpha spike was reduced by 24 % of its original height and at 6 min 45 sec by 40 %.

This experiment demonstrates that at a time when all the slowly-conducting fibres were blocked a proportion of the fast-conducting fibres was

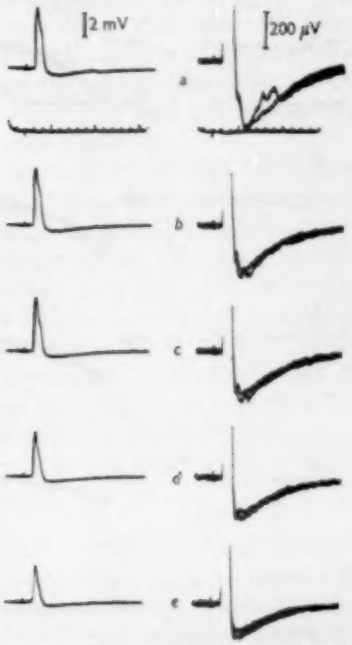



Fig. 2. Effect of 0·1 % procaine in modified Krebs–Henseleit solution on conduction in A fibres. Left-hand column, low amplification, to show fast-conducting fibres (alpha-beta), 5 sweeps superimposed. Right-hand column, high amplification, to show slowly-conducting fibres (delta), 10 sweeps superimposed; 5 of the sweeps made with stimulus intensity below threshold for slowly-conducting fibres. Records at the two different amplifications were made one immediately after the other; the time stated is that of the middle of the recording period. *a*, before application of procaine; *b*, *c*, *d*, *e*, 45 sec, 2 min 15 sec, 4 min 45 sec, 6 min 45 sec after application. Time marker, 1 and 5 msec intervals.

still conducting; it also shows that some of the fast-conducting fibres were blocked early, although subsequently the rate of blocking of this group was slower than that of the slowly-conducting fibres. This form of differential blocking, in which one or more groups of fibres tend to be blocked more rapidly than others and in which eventually all fibres are blocked, we shall refer to as *differential rate of blocking*.

It is of interest to note that when local anaesthetics are applied to spinal roots blocking of some of the fibres occurs just as quickly as it does when anaesthetics are applied to isolated single nerve fibres or to de-sheathed peripheral nerves, that is, within seconds of applying the solution.

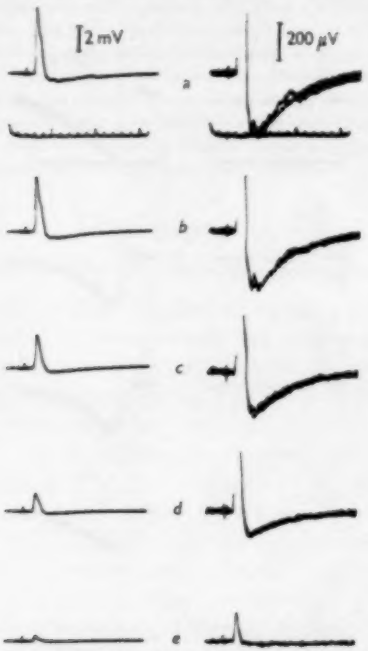



Fig. 3. Effect of 0·5 % procaine in modified Krebs–Henseleit solution on conduction in A fibres. Left-hand, low amplification, 5 sweeps superimposed. Right-hand, high amplification, 5 sweeps below threshold, 5 sweeps maximal for delta fibres. *a*, before application of procaine; *b*, 5–10 sec; *c*, 1 min 5 sec; *d*, *e*, 1 min 55 sec, 3 min after application. Time marker, 1 and 5 msec intervals.

The effect of applying a higher concentration is shown by the experiment illustrated in Fig. 3. Procaine 0·5 % in modified Krebs–Henseleit solution was applied to the same root as was used for the experiment illustrated in Fig. 2. By comparing Figs. 2 and 3 it is seen that 5–10 sec after applying this higher concentration of anaesthetic the delta wave was reduced by an amount similar to that occurring 45 sec after applying the weaker concentration. The alpha wave in Fig. 3, recorded within the next 5 sec, had already fallen by 12 %. The record taken at 1 min, with the higher concentration of anaesthetic, showed no trace of the delta wave, while the alpha wave, recorded a few seconds later, showed a reduction

of 46 % of its original height. At 1 min 55 sec the alpha wave was reduced by 74 % and by 3 min only a trace of it remained. It can be seen that, at a time when the height of the delta wave was reduced by similar amounts in the two experiments, with the low concentration of anaesthetic the reduction in the alpha wave was 4 % and with the high concentration it was 12 %. And when in both experiments the delta wave had gone, with the low concentration the reduction in the alpha wave was 8 % and with the high concentration it was 46 %. It is concluded from these two experiments and 11 others (9 with procaine and 2 with cocaine), in which the differential effect of two different concentrations of the same anaesthetic was examined on the same root, that the greatest degree of differential blocking occurs with weaker solutions.

In one respect the results obtained from applying the anaesthetics to spinal roots seem to differ from those observed when they are applied to peripheral nerves. Those working on peripheral nerves, for instance Matthews & Rushworth (1957), observed soon after the application of the anaesthetic a temporary phase during which there was blocking of most of the small fibres without blocking of many of the large fibres; this phase was followed eventually by blocking of all the large fibres. It was in this sense that Gasser & Erlanger (1929) stated 'small fibres were blocked before large ones'. But when the anaesthetic is applied to spinal roots, blocking of some large and of some small fibres occurs early and simultaneously, although subsequently the blocking of the fibres as a group still occurs at a differential rate.

### *Absolute differential blocking of fibre groups*

*Myelinated fibres.* The question next arises whether there are concentrations of anaesthetics that will selectively block all small fibres without blocking the larger fibres. The effects of applying a low concentration of anaesthetic to an anterior root and then increasing the concentration are illustrated in Fig. 4. The nerve to the hamstrings was stimulated, and recording was made from the first sacral anterior root. The conduction rates of the fast-conducting fibres was 70 m/sec (measured to the peak) and of the slowly-conducting fibres was from 18 to 35 m/sec. The first concentration of procaine in modified Krebs–Henseleit solution applied was 0·03 %. After this solution had surrounded the root for 21 min, the wave due to the slowly-conducting fibres was slightly modified in form and both its components showed some reduction in height; the latency to the foot of the wave was increased by 0·2–0·3 msec. After 22 min this solution was replaced by a 0·04 % solution. After this solution had been applied for 18 min, with no further changes of the slowly-conducted wave and no effect on the fast-conducted wave, it was replaced by a 0·05 % solution. After

this solution had surrounded the root for 15 min the height of the wave of the fast-conducting fibres still remained undiminished; there was a further slight alteration in form of the slowly-conducted wave and a further increase in its latency, but it may be seen that the majority of the fibres of this group were still conducting. When this solution was replaced 2 min later by a 0·06 % solution of procaine there was a rapid decrease in

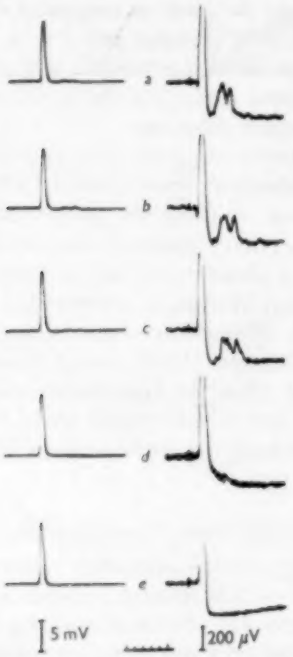



Fig. 4. Effect of 0·03, 0·05 and 0·06 % procaine in modified Krebs–Henseleit solution on conduction in A fibres. Each record consists of 5 superimposed sweeps. *a*, before application of procaine; *b*, 21 min after application of 0·03 % procaine; *c*, 15 min after application of 0·05 % procaine; *d*, 19 min after application of 0·06 % procaine; *e*, 62 min after application of 0·06 % procaine (120 min after application of the first procaine solution). Time marker 1 and 5 msec intervals.

amplitude of the slowly-conducted wave. Nineteen minutes after applying the 0·06 % solution only a trace of this wave was seen, whereas the fast-conducted wave remained unaltered. At the final observation, made 62 min after the application of the 0·06 % solution (120 min after the start of the experiment), there was no change in the fast-conducted wave; but all trace of the slowly-conducted wave had gone.

In this series of experiments, the concentration of the anaesthetic must have been adequate throughout the root to block all the smaller myelinated fibres present; yet such a concentration has failed to block the larger fibres. This form of differential blocking we shall refer to as *absolute differential blocking*.

The concentrations of anaesthetic that give absolute differential blocking of the gamma-delta group while leaving the alpha-beta group unaffected have been found to be fairly constant. The exact concentration, however, differs according to whether the solvent is Ringer–Locke solution or modified Krebs–Henseleit solution. The former solution behaves as if there is more procaine base available, which may be due to small differences in the pH of the two solutions. With regard to the concentrations needed to block the small fibres of the anterior roots without blocking the large fibres, it has been found that procaine at a concentration of 0·01 % in either solution has no effect on conduction of either group of fibres. At a concentration of 0·02 % slowing in the conduction rate of the gamma-delta group is seen, but there is no reduction in the amplitude of the action potential; there is no effect on conduction of the alpha-beta group. Procaine in Krebs–Henseleit solution at concentrations of 0·03–0·06 % causes progressively increasing slowing in conduction rate combined with blocking of the fibres of the gamma-delta group, the 0·06 % solution blocking all fibres of this group. These concentrations have no effect on the alpa-beta fibres. The corresponding concentrations of procaine in Ringer–Locke solution for blocking the gamma-delta group without affecting the alpha-beta group was found to be 0·04 % to 0·05 %.

When concentrations of anaesthetic are used that are just critical for a given group of fibres, following removal of the anaesthetic solution, recovery of conduction is rapid. For instance, in the experiment illustrated in Fig. 4, even though the small fibres had been blocked for 60 min, a proportion of them recovered within 2 min of replacing the anaesthetic by modified Krebs–Henseleit solution.

To determine the concentration of anaesthetic that is critical for blocking smaller delta fibres, experiments were done on the tail-nerve preparation; in these roots there is a group of delta fibres with a slower conduction rate than that of the delta fibres of the lumbar and first sacral anterior roots. An experiment typical of this series is illustrated in the left-hand column of Fig. 5. The conduction rate of the slowly-conducting group of delta fibres was 9·6–11·6 m/sec; the wave due to this group is seen as the large wave rising from the downstroke of the wave due to the fast-conducting fibres, the peak of which is off the record. Procaine 0·01 % in Ringer–Locke solution was applied. Within 2 min 40 sec the height of this wave had diminished by about 50 %; by 18 min the wave had gone.

Thus this concentration of procaine was critical or supra-critical for this group of small delta fibres; it did not affect conduction in the fibres with conduction rates around 40 m/sec. From experiments performed before this series on the more slowly-conducting delta fibres it had already been

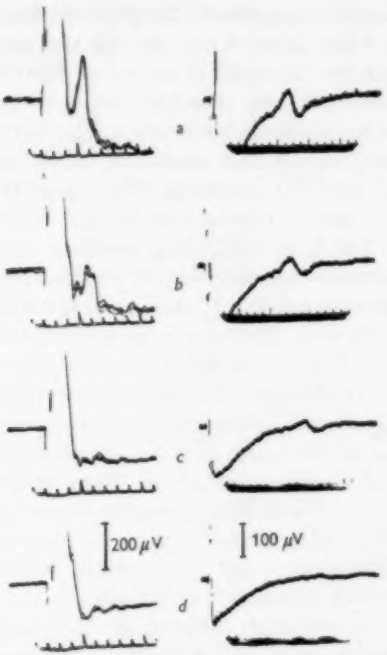



Fig. 5. Effect of 0·01 and 0·02 % procaine in Ringer–Locke solution on conduction in delta fibres and C fibres; delta fibres on left, C fibres on right. Delta fibres' component follows high-voltage spike of large fibres, which is off the trace at this amplification. Delta fibres record consists of 5 superimposed sweeps, C fibres record consists of single sweeps. *a*, before application of procaine; *b*, *c* (left-hand column), 2 min 40 sec, 18 min after application of 0·01 % procaine; (right-hand column), 2 min, 19 min after application of 0·01 % procaine; *d* (both columns), 30 sec after application of 0·02 % procaine. Time marker, 1, 5 and 20 msec intervals.

found that this concentration of procaine has no effect on conduction in larger, faster-conducting fibres.

Experiments on the tail-nerve preparation with cocaine gave similar results. It was possible to block all fibres with a conduction rate of 11–14 m/sec or less without blocking conduction of fibres conducting at rates around 40 m/sec. The concentration of cocaine that is just critical for these smaller fibres is 0·02 % in Ringer–Locke solution; concentrations slightly

higher than this, block fibres with conduction rates between 30 and 40 m/sec.

*Myelinated and C fibres.* The next question which arises is whether there are critical concentrations for the C fibres that block their conduction without affecting the myelinated fibres of the A group.

Before illustrating experiments that answer this question, mention must be made of the marked slowing of conduction rate that occurs in the C fibres with low concentrations of anaesthetics. This slowing in conduction rate is combined with blocking of many of the fibres. The combination of blocking and slowing in conduction rate causes an alteration in the form of the C fibres' action potential, and this makes a quantitative estimation less reliable than in the case of the myelinated fibres.

For the investigation of absolute differential blocking of C fibres, most experiments were done on the tail-nerve preparation; the concentrations of anaesthetic tested were those that we had found were critical for blocking conduction of the small myelinated fibres.

The effects of a low concentration of anaesthetic on conduction in the C fibres is illustrated in the right-hand column of Fig. 5. In this experiment, procaine 0·01 % in Ringer–Locke solution was first applied and it was followed later by a 0·02 % solution. The slowing of conduction rate of the C fibres can be seen: in the control records, the latency to the peak of the C fibres wave was 49 msec; after the root had been 2 min in procaine 0·01 %, it was 55 msec, and after 19 min, it was 64 msec. This slowing in conduction rate was associated with blocking of a large proportion of the fibres, as judged by the area of the wave. Increasing the concentration of procaine from 0·01 to 0·02 % caused complete block of all the C fibres.

To compare the concentrations of anaesthetic that are critical for C fibres and critical for small myelinated fibres, one may compare the records in the right- and left-hand columns of Fig. 5, for they are a typical example. It will be seen that procaine 0·01 % in Ringer–Locke solution blocked delta fibres with a conduction rate of 9·6–11·6 m/sec, and slowed conduction and blocked a large proportion of the C fibres. Most of the fibres of these two groups were blocked simultaneously. Doubling the concentration of procaine rapidly blocked the rest of the C fibres.

It was concluded from this and other experiments of the same type that concentrations of anaesthetic just sufficient to block the C fibres also block small delta fibres; but they are insufficient to block the larger delta fibres, or the gamma, beta or alpha fibres. The concentrations found that caused this absolute differential blocking were 0·01 % for both procaine and cocaine in Ringer–Locke solution.

In all experiments on the small myelinated and the C fibres it was found that the smallest, most slowly-conducting delta fibres present in the tail-

nerve preparation were blocked more rapidly than the C fibres. An experiment demonstrating this is illustrated in Fig. 6. In this tail-nerve preparation there were two groups of fibres with conduction rates in the delta range; their rates were 9–10 and 5–5·5 m/sec. They are seen as the two waves in the centre and later parts of the sweep following the wave due to the large fibres, the peak of which is off the record. The record made 3 min 50 sec after the application of procaine 0·01 % in modified Krebs–Henseleit

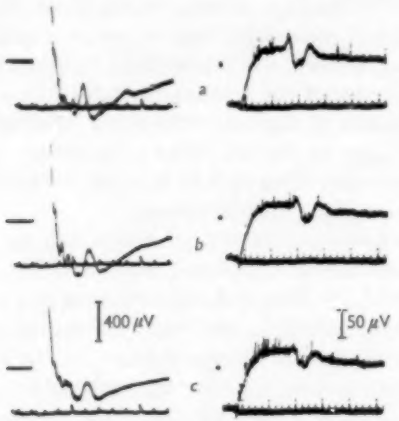



Fig. 6. Effect of 0·01 % procaine in modified Krebs–Henseleit solution on conduction in two groups of delta fibres and C fibres; delta fibres on left side, C fibres on right. Delta fibres record consists of 5 superimposed sweeps, C fibres record consists of single sweeps. *a*, before application of procaine; *b*, *c*, 3 min 50 sec, 5 min after application of procaine. Time marker, 1, 5 and 20 msec intervals.

solution shows all but complete obliteration of the wave of the most slowly-conducting delta fibres; it was abolished completely 5 min after application of the solution. By this time, the C fibres' action potential had been slightly altered in form and its latency increased from 47 to 53 msec. When the concentration of the procaine was increased to 0·02 %, the C fibres' action potential was rapidly abolished.

From such experiments it is seen that the generally accepted view of small fibres being blocked before large does not apply to a comparison between C fibres and the smallest myelinated fibres. For the smallest delta fibres are blocked with lower concentrations of anaesthetic than the C fibres; and when concentrations of anaesthetics adequate to block both groups are applied, the smallest delta fibres are blocked first.

It should be added that as the C fibres of the spinal roots are half the diameter of C fibres peripheral to the posterior root ganglia (Gasser, 1955).

it may well be that C fibres of peripheral nerves are not blocked with the same concentrations of anaesthetic and not in the same order with regard to the small myelinated fibres as the C fibres studied here. It may be that in peripheral nerves they are blocked simultaneously with larger delta fibres than those shown here and that they require higher concentrations of anaesthetic to block them.

## DISCUSSION

We have found, like most investigators of this problem, that small myelinated fibres are more susceptible to the action of local anaesthetics than large; this is manifest as a differential rate of blocking of the fibres. But we find that this order of blocking holds only within the group of A fibres. Non-myelinated fibres are not the most susceptible of all fibres to the action of local anaesthetics; the smallest fibres of the A group are blocked earlier and with lower concentrations of anaesthetic than the C fibres. It has been established in addition that there are critical concentrations of local anaesthetics that block conduction in fibres of one size while leaving fibres of larger size still conducting. This form of selective blocking we have called absolute differential blocking.

The disclosure of absolute differential blocking may be attributed to two factors, the application of the anaesthetic directly to the spinal roots and the use of low concentrations of anaesthetic. The absence of significant barriers to diffusion in the spinal roots allows the concentration of anaesthetic to become uniform fairly rapidly throughout the root.

The fact that some of the large fibres are blocked at the same time as the small fibres has been interpreted by various workers as indicating that myelinated fibres do not fall out on a fibre-size basis. In peripheral nerves, on which most previous work was done, the sheath is known to act as a barrier to diffusion. As the concentration of anaesthetic within the sheath rises small fibres would be blocked before large; but as the concentration of anaesthetics used by previous workers, including ourselves (Nathan & Sears, 1957), have been 'supra-critical' for all groups of fibres, the larger fibres would eventually also be blocked; though at any time a large proportion of smaller fibres and a small proportion of larger fibres would be blocked. That is the situation of differential rate of blocking. Further, local variations in the concentration of anaesthetic within the sheath, due to differences in the diffusion pathway for different nerve bundles, and also to the possibility of local damage to the sheath during the preparation of peripheral nerves, would also lead to blocking of some large fibres before all the small ones were blocked.

It is well known that during recovery from the effects of a local anaesthetic the small fibres recover last and the large fibres first. The fact that

this order is the reverse of that for blocking fibre groups constituted the final argument for Gasser & Erlanger (1929), that a hypothesis of differential blocking based on chemical combination of the anaesthetic with the protoplasm of the fibres is untenable; for, to quote their words, 'since the surface per unit volume increases directly as the diameter decreases, the smaller the fibre the greater the accessibility', and this would lead to small fibres being both blocked and unblocked before large. The fact of there being different blocking concentrations of anaesthetic for the different sizes of fibres explains the order of recovery; for when the anaesthetic is diluted a subcritical concentration for the large fibres is achieved before that for the small.

It is necessary now to relate what is known of the mechanism of the action of anaesthetics on conduction to the differences in structure and function of the different groups of fibres. The effect of a local anaesthetic on a single myelinated fibre of the toad, as shown by Tasaki (1953), is to reduce the amplitude of the action current. In whole nerves or roots this diminution in current would be manifest as a reduction in the amplitude of the externally recorded compound action potential. This in fact was found by Kato (1924) and by Davis, Forbes, Brunswick & Hopkins (1926), who showed that impulses being propagated through a region of uniformly narcotized nerve do so at diminished amplitude. The mechanism of the reduction in action current by the local anaesthetic has been attributed, in the giant squid axon, to a reduction in the number of sodium carriers (Shanes, Freygang, Grundfest & Amatniek, 1959; Taylor, 1959), and in Purkinje muscle fibres to inactivation of the sodium carrier at high membrane potential (Weidmann, 1955). It would seem reasonable to assume that the electrical properties of the membrane of small and large myelinated fibres are the same; and that under the action of a given concentration of local anaesthetic the action current developed per unit of membrane would be reduced by the same amount. This would mean that whatever possible differences exist between the local circuits of small and of large fibres, due to the different internodal resistances and to the different areas of nodal membrane developing action current, a local anaesthetic would produce the same proportionate reduction in the total nodal action current. The fact of absolute differential blocking with critical concentrations thus suggests that the ratio of the action current developed to that necessary for propagation is different in fibres of different sizes. This ratio—the safety factor—was measured by Tasaki (1953) in large single myelinated fibres of the toad. We know of no figures for the safety factor of smaller toad nerves nor of small or large mammalian fibres.

The experiments carried out to investigate differential blocking between

C fibres and A fibres showed that, with low concentrations of anaesthetics, the myelinated fibres conducting at rates of approximately 10 m/sec were blocked simultaneously with the C fibres; with still lower concentrations, the very smallest myelinated fibres were blocked at a time when a large proportion of the C fibres was still conducting, although their conduction rate was slowed. These observations on the fibres of the cat's spinal roots do not support the usual view that the C fibres are the most susceptible to the action of local anaesthetics. Attempts to correlate the sensory dissociation found on injection of anaesthetics in low concentrations in man with the differential blocking of fibre groups will need some re-assessment.

In a review of the action of local anaesthetics Toman (1952) reported that in experiments on the rabbit's vagus 'a 1 mM solution of procaine depresses A fibres first and C fibres last'. Everett & Toman (1954) concluded that such results 'do not support the older concepts of nerve block based on fibre size'. In the experiment on the rabbit's vagus, quoted by Toman, the comparison is being made between A and B fibres; yet it is known that these somatic and autonomic myelinated fibres show certain different electrical properties (Grundfest, 1939). Further, when comparisons are made between myelinated and C fibres, one is comparing the effects of anaesthetics on two different modes of conduction, on saltatory and continuous conduction; it might be expected that anaesthetics would affect them differently. When a comparison is made of fibres having the same electrical properties and the same mode of conduction, it is found that anaesthetics affect the fibres as though they are a continuous series differing only in size.

SUMMARY

1. The effects of local anaesthetics on conduction in myelinated and non-myelinated fibres of the spinal roots of the cat have been studied.

2. Different concentrations of anaesthetic are required to block conduction in fibres of different sizes; the concentration necessary to block small fibres was found to be lower than that necessary to block large fibres.

3. Differential rate of blocking of fibre groups was obtained when the concentration of anaesthetic exceeded that necessary for blocking all fibres present.

4. The minimum concentration of anaesthetic for blocking non-myelinated fibres was found also to block the smaller myelinated fibres.

5. Still lower concentrations of anaesthetic blocked the very smallest myelinated fibres without blocking the group of non-myelinated fibres.

We take this opportunity of thanking Drs E. A. Carmichael and W. A. Cobb for facilities to do this work and for their help.

## REFERENCES


DAVIS, H., FORBES, A., BRUNSWICK, D. & HOPKINS, A. McH. (1926). Studies of the nerve impulse. II. The question of decrement. *Amer. J. Physiol.* **76**, 448–471.

DIAMOND, J., GRAY, J. A. B. & INMAN, D. R. (1958). Depression of receptor potential in Pacinian corpuscles. *J. Physiol.* **141**, 117–131.

DIXON, W. E. (1905). Selective action of cocaine on nerve fibres. *J. Physiol.* **32**, 87–94.

ERLANGER, J. & GASSER, H. S. (1937). *Electrical Signs of Nervous Activity*. Philadelphia: University of Pennsylvania Press.

EVERETT, G. M. & GOODSELL, J. S. (1952). Greater resistance to procaine of slow fiber groups in some peripheral nerves. *J. Pharmacol.* **106**, 352.

EVERETT, G. M. & TOMAN, J. E. P. (1954). Procaine block of fiber groups in various nerves. *Fed. Proc.* **13**, 385.

GASSER, H. S. (1955). Properties of dorsal root on two sides of ganglia. *J. gen. Physiol.* **38**, 709–728.

GASSER, H. S. & ERLANGER, J. (1929). Role of fiber size in establishment of nerve block by pressure or cocaine. *Amer. J. Physiol.* **88**, 581–591.

GRUNDFEST, H. (1939). Properties of mammalian B fibers. *Amer. J. Physiol.* **127**, 252–262.

HEINBECKER, P., BISHOP, G. H. & O'LEARY, J. (1934). Analysis of sensation in terms of nerve impulse. *Arch. Neurol. Psychiat.* **31**, 34–53.

KATO, G. (1924). *Theory of Decrementless Conduction*. Tokyo: Nankado.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of medullated small-nerve fibers in mammalian ventral roots: efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

LEKSELL, L. (1945). Action potential and excitatory effects of small ventral root fibres to skeletal muscle. *Acta physiol. scand.* **10**, Suppl. 31.

MACINTOSH, R. (1957). *Lumbar Puncture and Spinal Analgesia*, 2nd ed. Edinburgh: E. and S. Livingstone.

MATTHEWS, P. B. C. & RUSHWORTH, G. (1957). Relative sensitivity of muscle nerve fibres to procaine. *J. Physiol.* **135**, 263–269.

NATHAN, P. W. & SEARS, T. A. (1957). Action of procaine on fibres of different calibre in spinal roots of cat. *J. Physiol.* **140**, 53–54*P*.

NATHAN, P. W. & SEARS, T. A. (1960*a*). Effects of phenol on nervous conduction. *J. Physiol.* **150**, 565–580.

NATHAN, P. W. & SEARS, T. A. (1960*b*). Differential nerve block by sodium-deficient solutions. *J. Physiol.* **154**, 41*P*.

SHANES, A. M., FREYGANG, W. M., GRUNDFEST, H. & AMATNIEK, E. (1959). Anaesthetic and calcium action in voltage-clamped squid giant axon. *J. gen. Physiol.* **42**, 793–802.

TASAKI, I. (1953). *Nervous Transmission*. Illinois: Charles C. Thomas.

TAYLOR, R. E. (1959). Effect of procaine on electrical properties of squid axon membrane. *Amer. J. Physiol.* **196**, 1071–1078.

TOMAN, J. E. P. (1952). Neuropharmacology of peripheral nerve. *Pharmacol. Rev.* **4**, 168–218.

VISOZO, A. D. & YOUNG, J. Z. (1948). Internode length and fibre diameter in developing and regenerating nerves. *J. Anat., Lond.*, **82**, 110–134.

WEIDMANN, S. (1955). Effects of calcium ions and local anaesthetic on electrical properties of Purkinje fibres. *J. Physiol.* **129**, 568–582.

*J. Physiol.* (1961), **157**, *pp.* 581–588
*With 5 plates and 1 text-figure*
*Printed in Great Britain*



# THE BASIS FOR SILVER STAINING OF SYNAPSES OF THE MAMMALIAN SPINAL CORD: A LIGHT AND ELECTRON MICROSCOPE STUDY

By E. G. GRAY and R. W. GUILLERY
*From the Department of Anatomy, University College London*

(*Received* 10 *February* 1961)

Neurofibrillae have been known to anatomists for many years (Schultze, 1862, 1871; Fromann, 1864). They can be stained by silver methods and often occur in axons, dendrites and nerve cell bodies. Also in some regions they mark the terminal parts of axons, where they form characteristic rings, clubs and reticulated structures, the classical 'boutons terminaux' (Cajal, 1911; Bartelmez & Hoerr, 1933; Bodian, 1937). Neurofilaments, fine osmiophilic threads 60–100 Å in diameter, have been described in electron micrographs by Schmitt & Green (1950), Fernández-Morán (1954), de Robertis & Bennet (1955) and Palay & Palade (1955). These authors have suggested that the neurofilaments form the basis of the neurofibrillae of light microscopists.

Boycott, Gray & Guillery (1960, 1961) have recently described neurofilamentous rings in the terminal bags in certain parts of the lizard brain, where neurofibrillar rings are found in great numbers. The present investigation was undertaken to determine whether similar neurofilamentous structures occur in the mammalian spinal cord, where the classical boutons are also plentiful. In addition, blocks stained with silver for terminal boutons by the classical methods of light microscopists have been examined by electron microscopy to determine whether silver is deposited on the neurofilaments and whether any other synaptic structures take up silver when such methods are used.

The presence of neurofilamentous rings in boutons was readily confirmed. The second question proved more difficult to answer, for it was impossible to obtain suitably fixed material for electron microscopy showing both neurofilaments and silver deposits in the same preparation. However, by a process of elimination other organelles could be excluded, leaving little doubt that, in the preparations used, the neurofilaments are in fact responsible for the silver deposits.

## METHODS

Several difficulties were encountered. Obtaining good fixation of spinal cord for electron microscopy was found to be a major problem. Perfusion techniques and thin slicing of frozen cord before immersion in fixative both proved unsatisfactory. The method (*b*) described below was finally adopted, but it entails the risk of mechanical damage. Another difficulty was to obtain silver-stained material sufficiently well fixed for electron microscopy. The ideal method, silver staining of osmium-fixed material, proved impossible. Method (*c*) (below) proved the most effective, although it will be seen that the electron micrographs of this material are much inferior to those obtained by conventional methods of osmium fixation.

*Light microscopy.* (*a*) Cats and rats were perfused with formol saline and the cords were stored in formol saline for 10 days. Blocks about 5–7 mm across were then impregnated by Bielschowsky's pyridine block method (see Romeis, 1948). A part of each block was then cut as frozen sections and the remainder of those blocks that showed successful impregnation were cut in two. One part was embedded in paraffin for light microscopy, and the other used for electron microscopy (see *c*). Other blocks were prepared by the block silver method of Cajal (see Wyckoff & Young, 1956) for light microscopy only.

*Electron microscopy.* (*b*) Slices of rat and cat cord were placed in 1 % osmium tetroxide in saline, buffered at pH 7·4 with veronal acetate. The slices were immediately chopped into very small pieces (preferably less than 0·3 mm thick in any direction) with a razor blade. They were transferred to fresh fixative and maintained for 3 hr with continuous agitation at approximately 4° C. Ethanol was used for dehydration, followed by staining for 3 hr in 1 % phosphotungstic acid (PTA) in absolute alcohol. The pieces were finally embedded in Araldite for sectioning (see Gray, 1959).

(*c*) The Bielschowsky material (see *a*) was cut into 0·2 mm slices to ensure good penetration of the stain and subsequently of the embedding medium. They were prepared for electron microscopy by ethanol dehydration and PTA staining and were embedded in Araldite (see *b*).

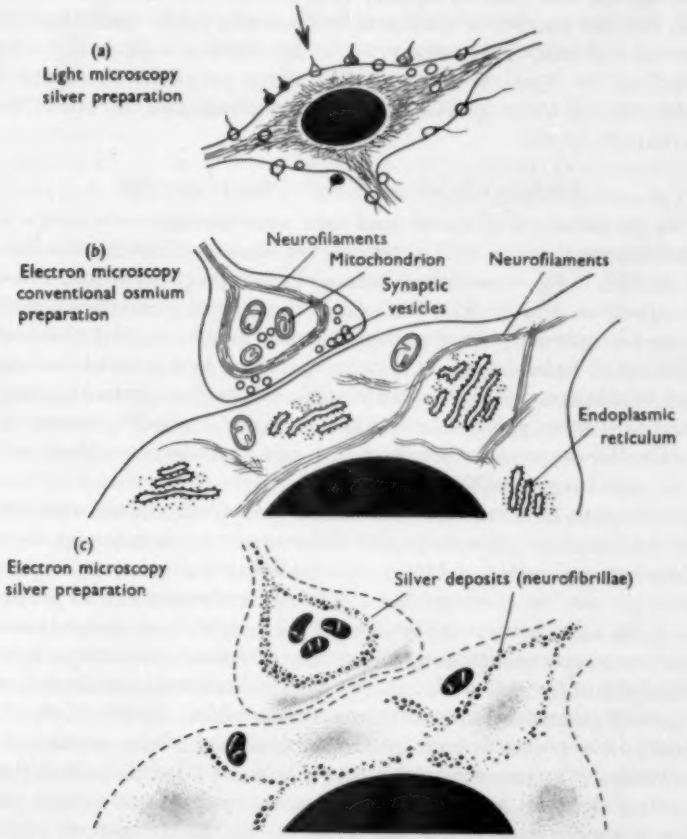
## RESULTS

### *Electron microscopy of osmium-fixed material*

The electron micrographs of the cat's spinal cord showed a number of terminal bags in which neurofilaments could be clearly observed (Text-fig. 1; Pl. 1; Pl. 2, fig. 1; Pl. 3, fig. 2). The terminal bags could be identified by their characteristic synaptic vesicles, about 500 Å in diameter, mitochondria and localized thickenings of the synaptic membranes. As far as can be determined by present techniques the neurofilaments of the terminal bag are structurally identical with the filaments that can be observed in myelinated axons (Pl. 1, $nf_1$), neuronal perikarya (see Palay & Palade, 1955) and some dendrites. Twenty to forty filaments are usually seen forming a bundle near the periphery of the synaptic bag. The cytoplasm containing the individual filaments is characteristically pale and free from synaptic vesicles. The bundles usually appear as profiles that can best be regarded as sections of rings or loops of filaments cut in various planes. A group of mitochondria occurs characteristically within the ring. Thus Pl. 1 shows most of the ring included in the section, whereas Pl. 3, fig. 2,

shows a ring cut radially and represented as two groups of dots, each dot being a sectioned filament.

Structurally these neurofilamentous rings are similar to the rings described in the lizard brain (Boycott *et al.* 1961) and it is reasonable to




Text-fig. 1. Diagrams of spinal cord synapses. In (*a*) ring-shaped, club-shaped (solid) and reticulated boutons are seen in silver preparations by light microscopy. The arrow indicates a ring bouton selected for illustration in (*b*) and (*c*) by electron microscopy.

suppose that in both situations the neurofilaments (or the pale cytoplasm in which they lie) take up silver stains to give the characteristic picture of ring-shaped terminal boutons (Pl. 5, fig. 1). Occasionally the filaments form an irregular network within the terminal bag (Pl. 2, fig. 1). It is

probable that such structures would appear as the reticulated or club-shaped endings of light microscopists (Text-fig. 1; Pl. 5, fig. 1).

The rat material that has been examined showed fewer neurofilaments in the terminal bags. Occasional endings with filaments could be found (Pl. 2, fig. 2). The pale cytoplasm (*pc*) around the filaments is clearly shown, but the number of filaments is markedly lower than that shown in the cat material. These observations by electron microscopy support the findings by light microscopy that silver preparations of rat cord generally show fewer rings than do similar preparations of cat cord (Phalen & Davenport, 1937).

*Electron microscopy of silver-stained material*

Silver preparations of spinal cord seen with the light microscope show the well known annular, club or reticulated boutons (Text-fig. 1). Plate 4, fig. 2, and Pl. 5, fig. 1, are taken from a block Cajal preparation, and show these structures clearly. The Bielschowsky material generally showed the boutons less intensely impregnated, but the morphology of the boutons was the same. In both types of silver preparation only some of the boutons appear continuous with the neurofibrillae of the preterminal axons. The cell bodies contain pale nuclei and these are surrounded by coarse or fine neuro-fibrillar aggregates (Pl. 5, figs. 1 and 2), which sometimes extend into the basal region of the dendrites.

Examination of ultra-thin sections of this silver-stained material by electron microscopy presents a quite different picture because (*a*) of greatly increased magnification and (*b*) many structures that are invisible by light microscopy can be observed because of their electron dense properties. Plate 4, fig. 1, is a low power electron micrograph of silver-stained material. Membrane structures are seen to be poorly preserved, but comparison with micrographs of the conventional osmium-fixed material (see above) makes the general organization readily understandable. In Pl. 4, fig. 1, the neuronal fibre plexus is seen (above) with a part of the perikaryon of a large ventral horn neurone with a row of boutons (*b*) at its surface (below). The silver deposits appear as dark strands or irregular masses that at higher magnifications (Pl. 3, fig. 1; Pl. 5, fig. 3) are seen to consist of discrete electron-dense particles, from 20–150 Å in diameter. In the fibre plexus (Pl. 4, fig. 1, and Pl. 3, figs. 1 and 3, at higher magnification) the silver can be clearly seen lying within the axoplasm and not in the myelin sheath (see Peters, 1955).

There are only two commonly occurring organelles in osmium preparations of myelinated axons of the spinal cord. They are the neurofilaments and the mitochondria (Pl. 1, $nf_1$ and $m_1$). In silver preparations the mitochondria appear as vague dense bodies, but they can nevertheless be

identified by their cristae. They show no impregnation at all (Pl. 3, figs. 1, 3, and Pl. 4, fig. 1). It thus appears probable that the silver is deposited upon the only other common known differentiated part of the axon, the neurofilaments (compare Meyer, 1957).

Observations on the nerve cell body confirm this. Fine or coarse strands of silver particles can be seen ramifying through the cytoplasm (Pl. 4, fig. 1, and Pl. 5, fig. 3). In electron micrographs of osmium-fixed material the neurofilaments show a similar distribution in the cell body (Palay & Palade, 1955). Again, the mitochondria of these regions appear entirely non-argyrophilic.

Finally, in Pl. 4, fig. 1, a row of boutons (*b*) is shown in contact with a nerve cell body. Two boutons are shown at higher magnification in Pl. 5, fig. 3. It is clear that neither the bouton cytoplasm, nor its surface membrane or mitochondria are argyrophilic. The synaptic vesicles, since they are membrane structures, are poorly preserved in the silvered material. A vague non-argyrophilic mass (*sv*?) can be seen near the synaptic membrane (in Pl. 5, fig. 3, for example). This mass might represent synaptic vesicles, but since rather similar masses appear in perikaryal cytoplasm (Pl. 4, fig. 1) it is more likely to be protein material precipitated by the formalin fixation. However, axosomatic boutons of mammalian cord invariably contain aggregates of synaptic vesicles, as is shown by osmium-fixed material (compare Pl. 3, fig. 2), while many boutons in the electron micrographs of silver preparations contain no silver particles in sufficient aggregations to be within the limits of resolution of light microscopy. So there can be little doubt that the synaptic vesicles are not responsible for the argyrophilia within a bouton.

The only remaining differentiated parts of the bouton that are known are the neurofilaments and their surrounding of pale cytoplasm. One or two strands of silver can be seen in boutons in Pl. 4, fig. 1 ($b_1$ and $b_2$). These could be oblique sections of rings. A more or less complete ring formed by silver deposits is shown in a bouton in Pl. 5, fig. 4. These, without doubt, correspond in position to the loops of neurofilaments. The fine silver particles clearly form linear aggregates (see Pl. 5, figs. 3 and 4), probably along the reaction product formed when the neurofilament (or the immediately adjacent cytoplasm) is fixed with formalin.

## DISCUSSION

Neurofilaments have been demonstrated by electron microscopy in the terminal presynaptic bags of axons of the spinal cord of the cat and rat. These filaments are often arranged to form rings that match the classical terminal boutons of light microscopy in shape and in approximate size. The

structures that can be seen in the terminal bag of spinal cord axons include surface membrane, mitochondria, synaptic vesicles and neurofilaments. The first three do not take up silver selectively in material impregnated for boutons nor do they have the right shape to form a possible basis for the neurofibrillar terminal boutons. It is thus highly probable that the silver is deposited in the neurofilamentous region of the synapse, probably on the fixation products of the filaments themselves.

Since terminal bags vary in the amount of neurofilamentous material that they contain it is relevant to ask whether this variation may have any functional significance. Several possibilities for further investigation arise. The number of neurofilaments may be related to size of terminal bag, number of mitochondria, or discharge activity of the axon. Thus in the lizard brain it was found that changes in the neurofilaments of boutons could be produced by altering environmental temperature, and these changes were accompanied by changes in the mitochondria in the same region (Boycott & Guillery, 1959; Boycott *et al.* 1960, 1961).

A great number of terminal bags show no neurofilaments in electron micrographs, fewer even in the rat than in the cat. While it is not possible without serial sections to state that they are absent in a given terminal, it is clear that many synaptic terminals have no neurofilaments. The variable effectiveness of the silver stains of light microscopy in different parts of the central nervous system and in different species is thus related to the presence or absence of neurofilaments in the synaptic terminal rather than to an inefficiency of the silver technique. On the other hand, mitochondria are almost invariably present in spinal cord synaptic terminals and this agrees with the observation that more synapses can be displayed by light microscopy with mitochondrial methods than can ever be seen with classical silver methods (Armstrong, Richardson & Young, 1956). It emphasizes that silver methods can never give more than a partial picture of synaptic organization.

## SUMMARY

1. Bundles of neurofilaments, often orientated in the form of a ring, can be observed by electron microscopy in a small proportion of the presynaptic bags of axon terminals of the spinal cord.

2. These neurofilaments form the basis of the argyrophilic material that constitutes the classical neurofibrillar rings and clubs of light microscopy. The possibility that other neuronal structures, the cytoplasm and surface membranes, myelin sheaths, mitochondria and synaptic vesicles, are argyrophilic can be excluded.

We are indebted to Professor J. Z. Young, F.R.S. for his interest and advice; to Mrs R. Tilly for photography; to Miss B. Shirra for technical assistance, and Miss J. de Vere for drawing the text-figure.

## REFERENCES

ARMSTRONG, J., RICHARDSON, K. C. & YOUNG, J. Z. (1956). Staining neural end feet and mitochondria after postchroming and carbowax embedding. *Stain Tech.* **31**, 263–70.

BARTELMEZ, G. W. & HOERR, N. R. (1933). The vestibular club endings in *Ameiurus*. *J. comp. Neurol.* **57**, 401–28.

BODIAN, D. (1937). The structure of the vertebrate synapse. A study of axon endings in Mauthner's cell and neighbouring centres in the goldfish. *J. comp. Neurol.* **68**, 117–60.

BOYCOTT, B. B. & GUILLERY, R. W. (1959). Environmental temperature and the reptilian nervous system. *Nature, Lond.*, **183**, 62–3.

BOYCOTT, B. B., GRAY, E. G. & GUILLERY, R. W. (1960). A theory to account for the absence of boutons in silver preparations of the cerebral cortex, based on a study of axon terminals by light and electron microscopy. *J. Physiol.* **152**, 3–5 *P*.

BOYCOTT, B. B., GRAY, E. G. & GUILLERY, R. W. (1961). Synaptic structure and its alteration with environmental temperature: a light and electron microscope study of the central nervous system of lizards. *Proc. Roy. Soc.* B, **154**, 151–172.

CAJAL, S. R. (1911). *Histologie du Système nerveux de l'Homme et des Vertébrés*, vol. I. Paris: Maloine.

DE ROBERTIS, E. D. P. & BENNET, H. S. (1955). Some features of the submicroscopic morphology of synapses of frog and earthworm. *J. biophys. biochem. Cytol.* **1**, 47–58.

FERNÁNDEZ-MORÁN, H. (1954). The submicroscopic structure of nerve fibres. *Prog. Biophys.* **4**, 112–47.

FROMANN, C. (1864). Über die Färbung der Binde- und Nervensubstanz des Rückenmarks durch Argentum Nitricum und über die Struktur der Nervenzellen. *Virchows Arch.* **31**, 129–51.

GRAY, E. G. (1959). Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat., Lond.*, **93**, 420–33.

MEYER, G. F. (1957). Submikroskopische morphologie von Gastropodennerven. *Z. Zellforsch.* **45**, 343–68.

PALAY, S. L. & PALADE, G. E. (1955). The fine structure of neurons. *J. biophys. biochem. Cytol.* **1**, 69–99.

PETERS, A. (1955). Experiments on the mechanism of silver staining IV. Electron microscope studies. *Quart. J. micr. Sci.* **96**, 317–22.

PHALEN, G. S. & DAVENPORT, H. A. (1937). Pericellular end-bulbs of the central nervous system of vertebrates. *J. comp. Neurol.* **68**, 67–81.

ROMEIS, B. (1948). *Mikroskopische Technik*. München: Leibniz.

SCHMITT, F. O. & GEREN, B. B. (1950). The fibrous structure of the nerve axon in relation to the localisation of neurotubules. *J. exp. Med.* **91**, 499–504.

SCHULTZE, M. S. (1862). Untersuchungen über den Bau der Nasenschleimhaut namentlich die Struktur und Endigungsweise der Geruchsnerven. *Abh. Naturf. Ges. Halle*, **7**, 1–87.

SCHULTZE, M. S. (1871). Allgemeines über die Struckturelementes des Nervensystems. *Handbuch der Lehre von den Geweben des Menschen und der Tiere*. ed. S. Stricker, pp. 108–36. Leipzig: Engelmann.

WYCKOFF, R. W. G. & YOUNG, J. Z. (1956). The motor-neuron surface. *Proc. Roy. Soc.* B, **144**, 440–50.

## EXPLANATION OF PLATES

In all plates letters indicate structures thus: *b*, bouton; *m*, mitochondrion; *my*, myelin sheath; *nb*, neurofibrillae; *nf*, neurofilaments; *pc*, pale cytoplasm containing neurofilaments; *post*, post-synaptic cytoplasm; *si*, silver deposits; *sv*, synaptic vesicles.

### PLATE 1

Fig. 1. Electron micrograph. Cat cord. A presynaptic bag containing a ring of neurofilaments surrounding a group of mitochondria; synaptic vesicles are also present. (Below) myelinated axon.

PLATE 2

Fig. 1. Electron micrograph. Cat cord. Presynaptic bag containing an irregular arrangement of neurofilaments.

Fig. 2. Electron micrograph. Rat cord. Presynaptic bag containing a few neurofilaments situated in a clear zone of the cytoplasm.

PLATE 3

Fig. 1. Electron micrograph. Cat cord. Silver deposits within a myelinated axon.

Fig. 2. Electron micrograph. Cat cord. Dots within the circles represent a radial section of a ring of neurofilaments.

Fig. 3. Electron micrograph. Cat cord. Silver deposits within a myelinated axon.

PLATE 4

Fig. 1. Electron micrograph. Cat cord. Silver-stained preparation. Fibre plexus (above) and large ventral horn cell (below) with a row of boutons.

Fig. 2. Low-power light micrograph. Cat cord. Cajal silver preparation of large neurone with boutons.

PLATE 5

Fig. 1. Light micrograph. Cat cord. Cajal silver preparation of large neurone with boutons and fibre plexus.

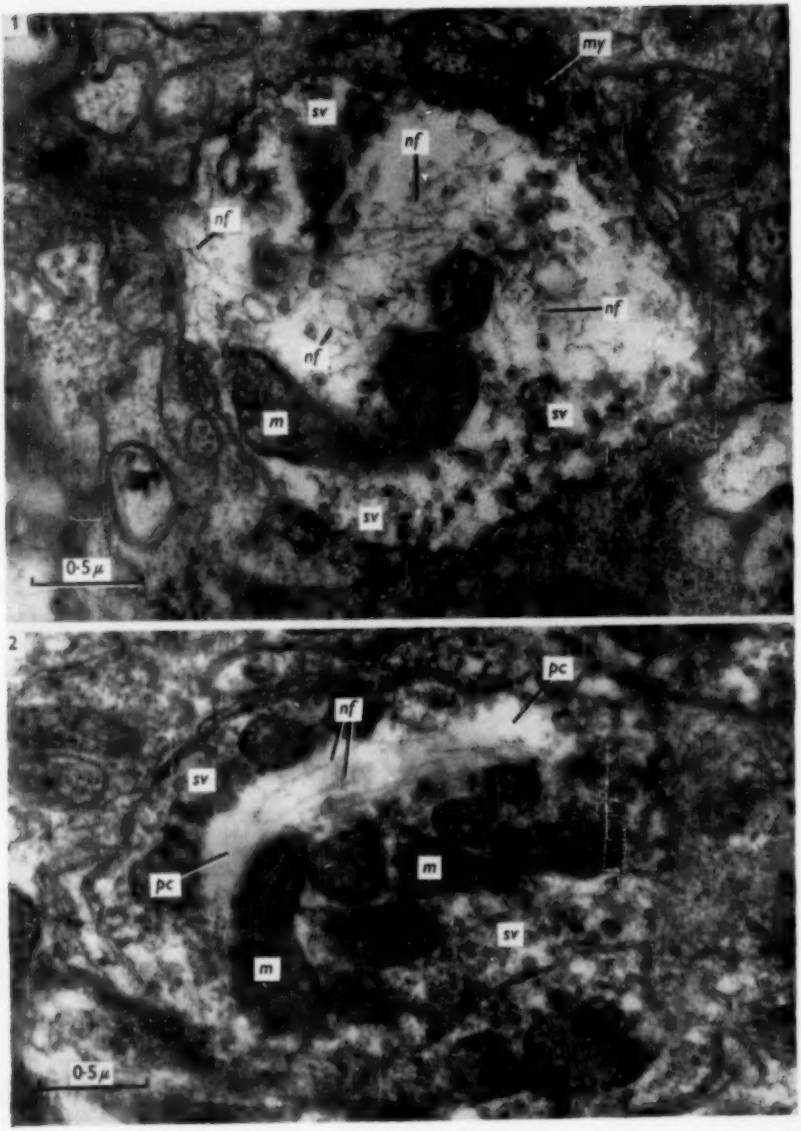
Fig. 2. Light micrograph. Cat cord. Neurone with neurofibrillae extending into basal regions of dendrites.

Fig. 3. Electron micrograph. Cat cord. Axo-somatic boutons in a silvered preparation.

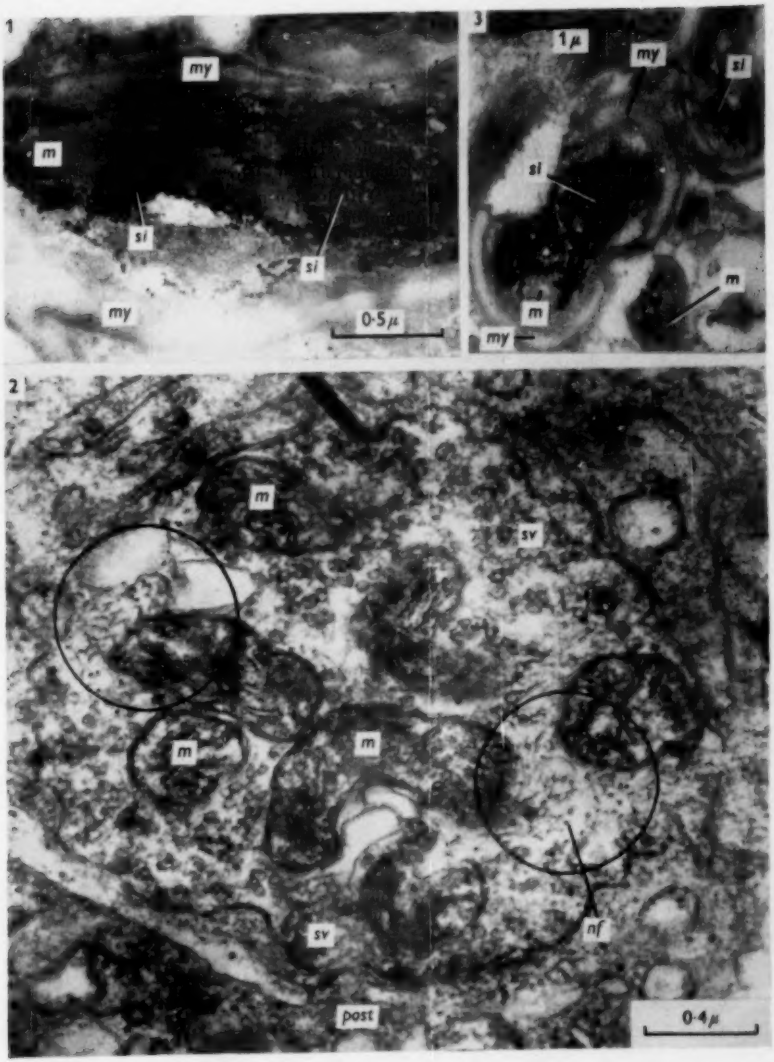
Fig. 4. Electron micrograph. Cat cord. Axo-somatic bouton showing ring of silver particles.

E. G. GRAY AND R. W. GUILLERY

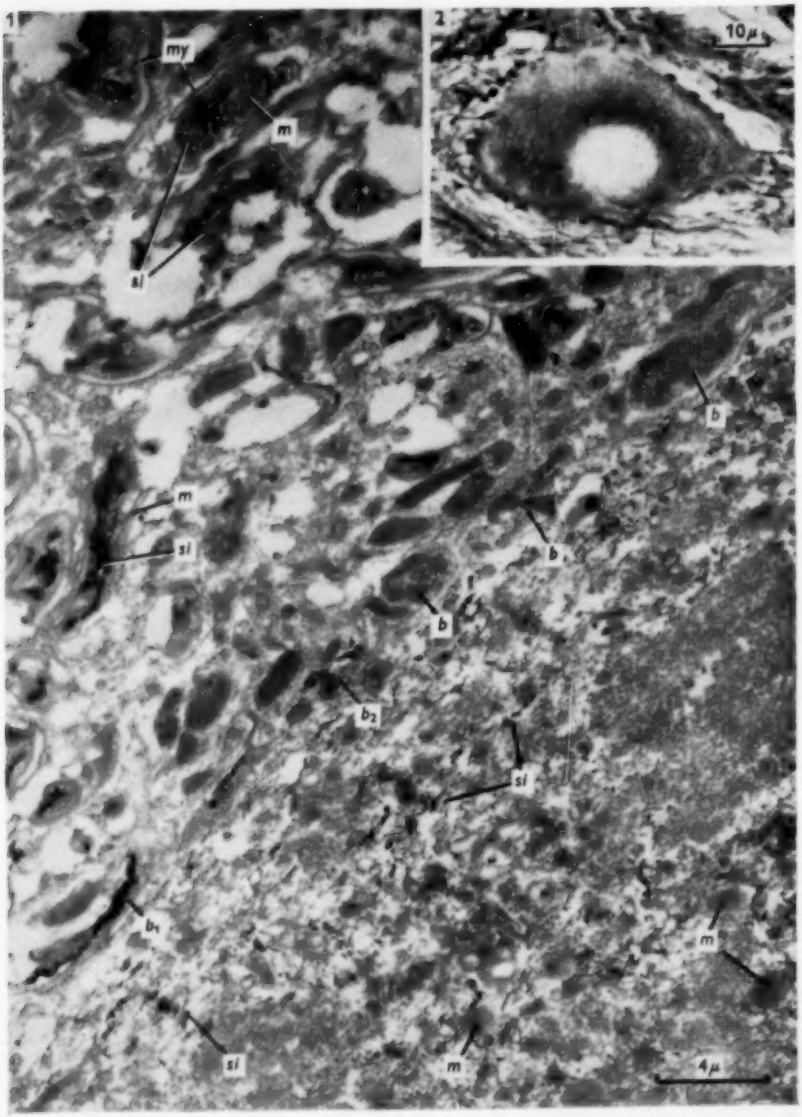
(*Facing p.* 588)




E. G. GRAY AND R. W. GUILLERY




E. G. GRAY AND R. W. GUILLERY




E. G. GRAY AND R. W. GUILLERY

1
10μ
2
nb
20μ
si
m
m
b
4
1μ
si
m
post
0·5μ
3
sv?
b
m
sv?
si
si
si
m
m
m

*J. Physiol.* (1961), **157**, *pp.* 589–602
*With 8 text-figures*
*Printed in Great Britain*



# BRADYKININ AS A VASODILATOR IN MAN

By R. H. FOX, R. GOLDSMITH, D. J. KIDD and G. P. LEWIS

*From the Division of Human Physiology, and Division of Physiology and Pharmacology, National Institute for Medical Research, London, N.W. 3*

(*Received* 13 *February* 1961)

The name bradykinin was first used by Rocha e Silva, Beraldo & Rosenfeld (1949) to describe a slow-acting smooth-muscle-stimulating polypeptide obtained by incubation of snake venom or trypsin with plasma proteins. The enzyme forming bradykinin has been found in a number of animal tissues and in particular in most of the exocrine glands. It is thought that bradykinin is responsible for the functional vasodilatations in the salivary gland of the cat (Hilton & Lewis, 1955, 1956), and in the human eccrine sweat gland, where the vasodilatation may also play an important role in temperature regulation (Fox & Hilton, 1958). Bradykinin has also been suggested as a mediator in the early stages of the inflammatory response (Hilton & Lewis, 1957). Recently the pure peptide has been isolated and its structure elucidated, and it has been synthesized (Elliott, Lewis & Horton, 1960*a*, *b*; Boissonnas, Guttman, Jaquemond, Konzett & Stürmer, 1960). The pure peptide has a powerful vasodilator action in cats, stimulates certain types of smooth muscle, increases capillary permeability and produces pain when applied to a blister base (Elliott, Horton & Lewis, 1960).

In the present experiments bradykinin has been shown to be a most powerful vasodilator substance in man. It has been administered intra-arterially and intravenously, and its actions have been compared to those of other vasodilator compounds.

## METHODS

The experiments were performed on nineteen healthy adult males. Bilateral hand or forearm blood flows were measured by venous occlusion plethysmography with the subjects seated in a stirred and temperature-controlled water-bath, following the technique described by Edholm, Fox & Macpherson (1956). Changes in the blood flow in the skin of the face, neck or forearm were recorded using surface calorimeters. The calorimeters consist of a heat-flow disk (Hatfield, 1950) enclosed in a Perspex capsule, and were irrigated with water at 32° C. The drugs were either administered as single doses in 1 ml. of 0·9 % NaCl solution and injected in 5 sec at a fixed time during the venous cuff-inflation cycle, or by continuous infusion employing a Palmer slow-injection apparatus. The natural bradykinin was prepared by the procedures described by Elliott, Horton *et al.* (1960); the molecular weight of

the dihydrochloride is 1131. The responses to injections of natural bradykinin were compared with those to synthetic bradykinin, which was kindly supplied by Sandoz Ltd, histamine acid phosphate (expressed as base), acetylcholine chloride, isoprenaline sulphate, and 5-hydroxytryptamine creatinine sulphate. Intra-arterial injections were made into the brachial artery at the elbow, and intravenous injections into the antecubital vein. The vasodilator response to each drug injection was obtained by subtracting the pre-injection blood flow, as given by the mean of the two inflows immediately preceding the injection, from the peak blood flow recorded following the injection. Pulse rates were counted from the blood flow traces.

## RESULTS

Intra-arterial injection or infusion of those compounds which caused large increases in blood flow were followed by a feeling of swelling and warmth of the forearm. The magnitude of these sensations was not dependent on the particular compound injected but on the size of the vasodilatation elicited. With intra-arterial injections the largest doses of all the drugs injected sometimes caused transient pain or unpleasant sensation deep in the forearm.

### *Intra-arterial injections of drugs on total forearm blood flow*

The vasodilatation produced by natural bradykinin was compared with the responses to injections of synthetic bradykinin, histamine and isoprenaline in doses ranging from 0·05 to 5·0 $\mu$g. Responses to the same doses, on a weight-for-weight basis, were directly compared to provide a quantitative comparison. There was no difference in potency between natural and synthetic bradykinins. Their potency was about the same as that of isoprenaline but greater than that of histamine ($P < 0{\cdot}001$). The results are given in Table 1.

In other experiments the effects on forearm flow of doses of acetylcholine 0·5–250 $\mu$g and 5-hydroxytryptamine 0·5–5·0 $\mu$g were measured. In order to obtain increases in blood flow similar to those produced by bradykinin much greater amounts of acetylcholine had to be injected. There was no response to 0·5 $\mu$g 5-hydroxytryptamine, but doses of 1·0–5·0 $\mu$g always produced a small vasodilatation.

Figure 1 shows a comparison of the potencies of natural bradykinin, acetylcholine and histamine, and that bradykinin is the most and acetylcholine the least potent substance. Further comparisons of the drugs are shown in Fig. 4. The vasodilatation produced by bradykinin was resistant to intra-arterial mepyramine (Fig. 1) and also was unaffected by an intra-arterial dose of atropine sulphate 0·6 $\mu$g.

The times taken to reach maximum vasodilatation following injections of bradykinin and isoprenaline were greater than those for acetylcholine and histamine (Fig. 2). In one experiment it was possible to examine the action of both bradykinin and histamine during a post-fainting state. The

subject was a student who had a history of fainting, which was revealed subsequently. On insertion of the arterial needle he fainted and became unconscious for a short time. After regaining consciousness he said he was willing to continue the experiment. When the arterial needle was reinserted he again felt faint but this time did not lose consciousness. The injections

TABLE 1. Forearm vasodilator responses to intra-arterial injections of vasoactive compounds. The pre-injection resting blood flow and the maximum increase above the resting value are shown (ml./100 ml. tissue/min). Values for different compounds on the same line are directly comparable as they were obtained close in time in the same experiment

| | | Natural bradykinin | | Synthetic bradykinin | | Histamine | | Isoprenaline | |
|---|---|---|---|---|---|---|---|---|---|
| Subject | Dose (μg) | Resting flow | Maximum increase | Resting flow | Maximum increase | Resting flow | Maximum increase | Resting flow | Maximum increase |
| A | 1·0 | 4·7 | 17·0 | — | — | 5·1 | 13·8 | — | — |
| B | 0·5 | 4·2 | 12·4 | — | — | 3·6 | 9·8 | — | — |
| C | 0·05 | 2·9 | 3·9 | — | — | 3·6 | 2·2 | — | — |
| | 0·5 | 4·4 | 5·2 | — | — | 2·9 | 4·2 | 3·7 | 2·8 |
| | 1·0 | 5·8 | 6·3 | — | — | 3·6 | 4·3 | — | — |
| | 3·0 | 4·9 | 8·0 | — | — | 3·2 | 6·3 | 3·7 | 6·1 |
| | 5·0 | 5·5 | 8·0 | — | — | 4·6 | 7·2 | — | — |
| D | 0·1 | 2·9 | 4·1 | — | — | 2·7 | 4·3 | — | — |
| | 0·5 | 2·9 | 7·3 | — | — | 3·3 | 5·1 | — | — |
| | 5·0 | 2·6 | 10·0 | — | — | 4·2 | 7·7 | — | — |
| E | 0·1 | 3·3 | 3·4 | — | — | 3·3 | 0·7 | — | — |
| | 1·0 | 3·2 | 7·3 | — | — | 3·3 | 5·4 | 2·7 | 8·4 |
| | 3·0 | 3·8 | 14·4 | — | — | 3·6 | 8·7 | 5·5 | 6·8 |
| | 5·0 | 3·6 | 16·0 | — | — | 3·6 | 10·6 | — | — |
| F | 0·5 | 4·2 | 6·6 | 4·4 | 7·0 | — | — | 3·4 | 7·0 |
| | 1·0 | 2·2 | 8·1 | 2·6 | 7·8 | — | — | 2·3 | 7·4 |
| | 3·0 | 2·4 | 12·8 | 5·6 | 18·6 | — | — | 2·4 | 8·4 |
| G | 0·1 | 1·1 | 15·0 | 1·2 | 7·2 | — | — | — | — |
| | 0·5 | 2·3 | 8·2 | 1·5 | 10·4 | — | — | 1·2 | 9·5 |
| | 1·0 | 2·0 | 12·0 | 1·8 | 12·9 | — | — | 2·1 | 8·8 |
| | 1·0 | 1·5 | 12·9 | — | — | 1·3 | 9·2 | — | — |
| | 3·0 | 1·5 | 15·3 | 2·2 | 14·6 | — | — | 0·8 | 11·1 |
| | 3·0 | 2·6 | 12·8 | — | — | 1·4 | 8·6 | — | — |
| H | 1·0 | 3·4 | 8·2 | — | — | 3·7 | 5·6 | — | — |
| | 1·0 | 3·4 | 10·2 | — | — | — | — | 1·8 | 13·3 |

were begun when the pulse rate had returned to 60 beats/min. The responses to both bradykinin and histamine were much reduced during the post-fainting state but returned to nearly normal during the experiment. This experiment is illustrated in Fig. 3.

*Intra-arterial injections of drugs on forearm skin blood flow*

In most experiments the changes in skin blood flow were measured simultaneously with total blood flow in the same forearm (see Fig. 4). As the measurements of change in the skin flow cannot be converted into absolute rates of blood flow, an accurate quantitative comparison of the

responses in skin and total forearm blood flow was not possible, yet it was found that bradykinin, histamine and acetylcholine produced the same relative changes in blood flow of the skin and of the whole forearm. Reddening of the forearm with the larger doses of all three compounds provided visual evidence of cutaneous vasodilatation.

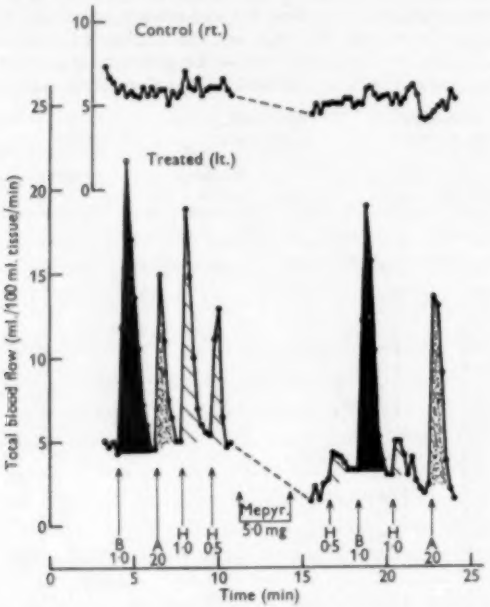



Fig. 1. Forearm vasodilator responses to intra-arterial injections of natural bradykinin (B), acetylcholine (A) and histamine (H), before and after giving 5·0 mg mepyramine intra-arterially. (All other doses in $\mu$g.)

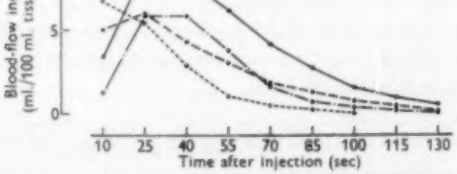



Fig. 2. The time course of the vasodilatation following injections of natural bradykinin, histamine, acetylcholine and isoprenaline. Each curve represents the mean response for all injections of that compound: —— bradykinin; —.— isoprenaline; — — — histamine; ····· acetylcholine.

In the post-fainting state in which the sensitivity of the forearm flow to bradykinin and histamine was reduced, that of the skin blood flow was reduced to an even greater extent (Fig. 3).

Arterial injection of 5-hydroxytryptamine decreased the skin flow in spite of a slight increase in total forearm flow (Figs. 4 and 5).

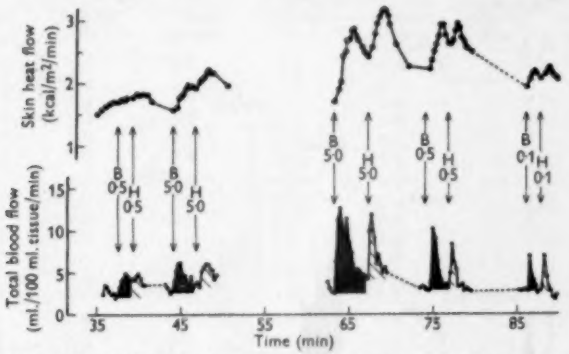



**Fig. 3.** Skin and total forearm blood flow responses to intra-arterial injections of natural bradykinin (B) and histamine (H) in the post-fainting state. The subject first fainted at zero time and nearly fainted a second time at 30 min. (All doses in $\mu$g.)

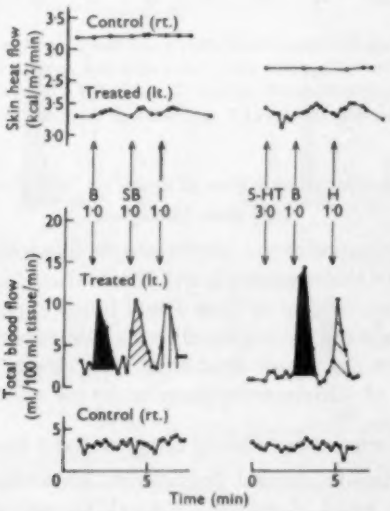



**Fig. 4.** Skin and total forearm blood flow responses of two subjects to intra-arterial injections of natural bradykinin (B), synthetic bradykinin (SB), isoprenaline (I), and 5-hydroxytryptamine (5-HT). (All doses in $\mu$g.)

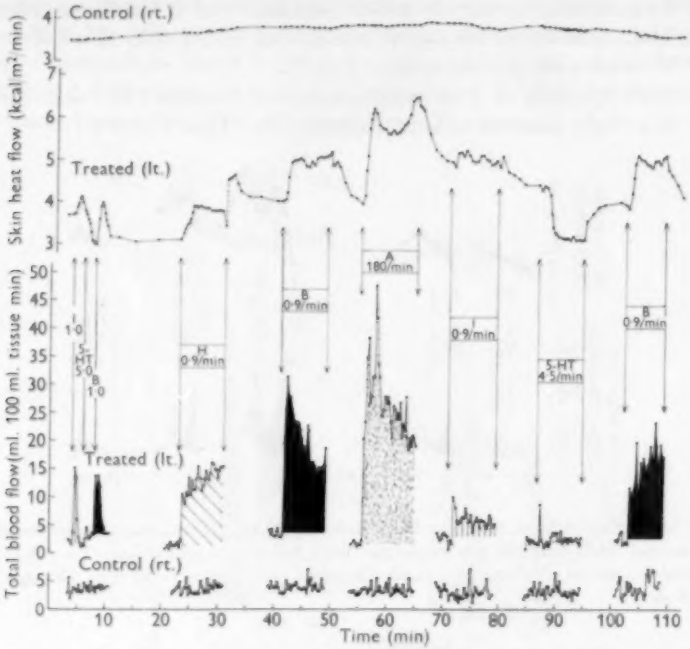



Fig. 5. Skin and total forearm blood flow responses to intra-arterial infusions of natural bradykinin 0·9 μg/min (B), histamine 0·9 μg/min (H), acetylcholine 180 μg/min (A), isoprenaline 0·9 μg/min (I) and 5-HT 4·5 μg/min. The results of single injections of isoprenaline (1·0 μg), 5-HT (5·0 μg) and bradykinin (1·0 μg) are also given.

### *Intra-arterial infusion of drugs on total forearm and skin blood flow*

One of the two experiments performed is illustrated in Fig. 5. The relative potencies of the compounds and the relationship between skin and total blood flow were similar to those found in the experiments with single injections. The response to isoprenaline in this experiment was smaller than expected, but the figure illustrates very clearly the striking vasoconstrictor action of 5-hydroxytryptamine on the skin.

### *Intra-arterial injection of drugs on hand blood flow*

In three experiments natural bradykinin, histamine and isoprenaline were injected in equal doses (0·1–5·0 μg) to provide a quantitative comparison. These results are shown in Table 2 and Fig. 6. Unlike the results obtained on the forearm flow, histamine was more active than

TABLE 2. Hand vasodilator responses (ml. blood/100 ml. tissue/min) to intra-arterial injections. The pre-injection resting blood flow and the maximum increase above the resting value are shown. Values for different compounds on the same line are directly comparable as they were obtained close in time

| | | Natural bradykinin | | Histamine | | Isoprenaline | |
|---|---|---|---|---|---|---|---|
| Subject | Dose (μg) | Resting flow | Maximum increase | Resting flow | Maximum increase | Resting flow | Maximum increase |
| G | 0·1 | 4·5 | 1·1 | 2·6 | 4·8 | 1·2 | 6·0 |
| | 0·5 | 2·2 | 2·5 | 1·3 | 6·1 | — | — |
| | 1·0 | 1·0 | 9·7 | 3·1 | 7·9 | — | — |
| | 1·0 | 1·8 | 4·7 | 1·8 | 5·4 | — | — |
| | 3·0 | 2·3 | 9·5 | 2·7 | 8·0 | 3·6 | 3·8 |
| | 5·0 | 1·0 | 8·4 | 1·5 | 12·4 | — | — |
| H | 0·1 | 5·3 | 0·9 | 4·0 | 6·7 | 4·3 | 4·3 |
| | 0·5 | 4·2 | 2·7 | 2·9 | 9·5 | — | — |
| | 1·0 | 3·6 | 8·8 | 3·5 | 12·2 | — | — |
| | 1·0 | 5·5 | 10·2 | 4·3 | 18·6 | — | — |
| | 3·0 | 3·3 | 20·6 | 3·0 | 21·8 | 6·6 | 14·4 |
| | 3·0 | 3·7 | 14·2 | 3·9 | 15·7 | — | — |
| | 5·0 | 4·2 | 11·1 | 4·3 | 15·3 | — | — |
| I | 0·1 | 4·3 | 6·5 | 4·6 | 6·2 | — | — |
| | 0·5 | 5·4 | 7·0 | 4·5 | 12·8 | — | — |
| | 0·5 | 5·6 | 11·5 | 3·3 | 16·0 | 6·1 | 6·9 |
| | 1·0 | 3·5 | 13·4 | 2·0 | 16·1 | — | — |
| | 1·0 | 5·5 | 16·2 | 5·1 | 15·8 | 5·6 | 12·5 |
| | 3·0 | 5·3 | 23·9 | 6·4 | 17·2 | — | — |

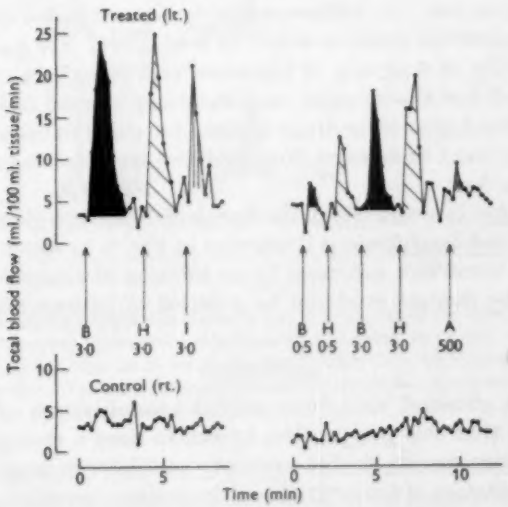



Fig. 6. Hand vasodilator responses to intra-arterial injections of natural bradykinin (B), histamine (H), isoprenaline (I) and acetylcholine (A). (All doses in μg.)

bradykinin ($P < 0.01$). Acetylcholine (10–500 $\mu$g) had only a small vasodilator activity—it was even less active than in the forearm.

When the vasodilator responses to histamine were compared in the hand and forearm (see Tables 1 and 2) the mean maximum increases were found to be greater in the hand than in the forearm ($P < 0.02$). For instance, the mean increases to 0·5 $\mu$g were 11·1 for the hand but only 3·4 for the forearm, and for 3 $\mu$g the corresponding values were 15·7 and 7·9.

### *Intravenous administration of drugs*

With intravenous injections of small doses of bradykinin the first effect was either a sensation of warmth in the face or a visible reddening of the face and neck. The minimum single intravenous dose required to produce any observable effect was found in four subjects to be between 0·1 and 0·4 $\mu$g/kg body weight. With larger doses (1 $\mu$g/kg or more) the feeling of warmth spread to the limbs and the rest of the body. Other sensations reported were a feeling of pulsations of the lips, cheeks and roof of the mouth, a dry or metallic taste on the tongue or an intense but indescribable taste in the mouth, awareness of cardiac action and catching of breath. The feeling of warmth and the appearance of flushing were most pronounced in the face and neck, and the feeling of bodily warmth was followed by that of coldness.

*Effect on blood flow.* On intravenous injection or infusion histamine was found to be about six times as active as bradykinin. For example, intravenous infusions of 9 $\mu$g/min of histamine and 53 $\mu$g/min of bradykinin produced small but almost equal vasodilatations in hand and forearm. It was further found that these drugs in doses too small to cause an appreciable effect on hand or forearm flow produced large increases in the face and neck skin flow.

This difference in sensitivity of the vessels of different regions of the body to histamine and bradykinin is illustrated in Fig. 7, in which the changes in peripheral blood flow produced by an infusion of bradykinin are compared with the changes produced by a period of indirect heating.

## DISCUSSION

The results obtained with intra-arterial administration of bradykinin to man show that this polypeptide, known to have a strong vasodilator action in various species, is also a potent vasodilator in man. A diagrammatic representation of the activities of the various vasodilator substances injected intra-arterially is given in Fig. 8.

This comparison is made on a weight-for-weight basis. However, on a molar basis bradykinin is many times more active on forearm and hand

flow than any of the other substances examined, because the molecular weight of the dihydrochloride of bradykinin which was used for these experiments is 1131 as compared with those of histamine base and isoprenaline sulphate which are 101 and 307 respectively.

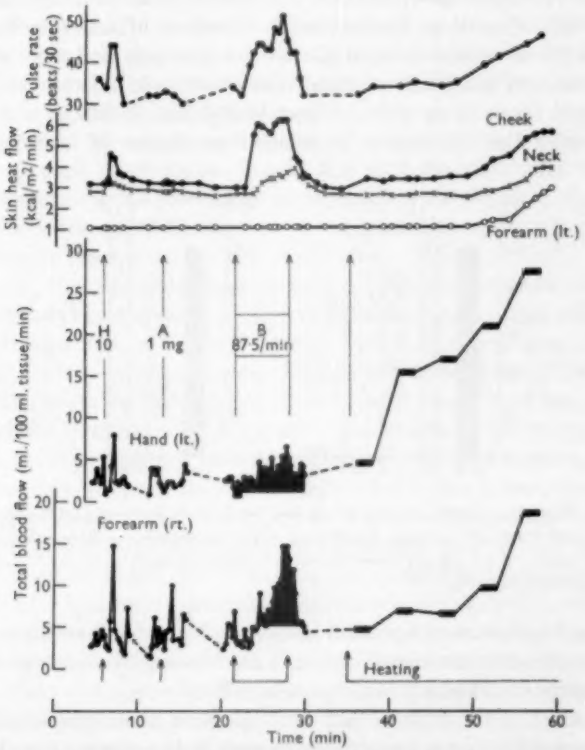



Fig. 7. Vasodilator responses in the hand and forearm and in the skin of the cheek and neck following intravenous injections of histamine 10 $\mu$g (H) and acetylcholine 1·0 $\mu$g (A), and an infusion of natural bradykinin 87·5 $\mu$g/min (B). The vasodilator effect of indirect heating is also shown. Heat-flow symbols: ● = cheek; × = neck; o = forearm (left).

The findings that, first, on a weight basis intra-arterial injection of bradykinin is more powerful than histamine in the forearm but somewhat less so in the hand and, secondly, on intravenous administration histamine is much more effective than bradykinin in both forearm and hand, can be explained by the fact that histamine is scarcely inactivated or destroyed in

human blood (Best & McHenry, 1930; Anrep, Barsoum & Ibrahim, 1947), whereas bradykinin is relatively rapidly destroyed (Lewis, 1960).

An example of how the distance from the site of an arterial injection to the site of action will affect the response to a substance which is rapidly destroyed in blood is well illustrated by Duff, Greenfield, Shepherd & Thompson (1953) for acetylcholine. They found that the magnitude of the response in the hand to intra-arterial injections of acetylcholine varied directly with the distance from the site of injection and they attributed this difference to the inactivation of acetylcholine in blood. Acetylcholine is destroyed much more quickly than bradykinin in blood, and this also explains why the differences in relative potencies of acetylcholine on

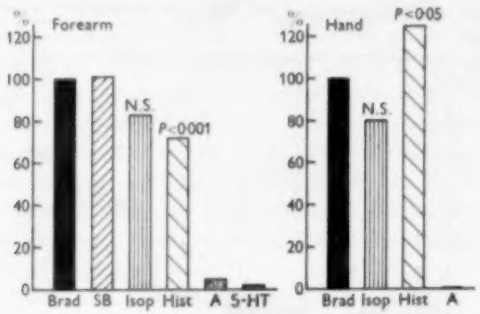



Fig. 8. The vasodilator activity of natural bradykinin in hand and forearm compared with those of synthetic bradykinin (SB), histamine and isoprenaline, based on the means of the paired measurements. Values for acetylcholine (A) and 5-HT are approximate only.

arterial and intravenous injection are so much greater than those of bradykinin. On intravenous injection even 1 mg of acetylcholine was ineffective in producing vasodilation in forearm and hand.

In contrast to the finding that bradykinin is considerably more active than acetylcholine as a vasodilator in man is the observation by Elliott, Horton *et al.* (1960) that in the cat bradykinin and acetylcholine are equiactive. This apparent discrepancy can again be explained by different rates of destruction in blood, as Hawkins & Mendel (1947) found that acetylcholine is destroyed much more rapidly in human blood than in cat blood, whereas bradykinin is destroyed almost equally in both species (G. P. Lewis, unpublished).

In addition to differences in the rates of destruction there may be differences in selectivity of action of the drugs on particular parts of the vascular bed. These differences may be due to the fact that both the sympathetic innervation and the vascular structure vary greatly between

hand and forearm. For instance, the ratio of skin to muscle tissue is much larger in the hand than in the forearm and an increase in the blood flow to the hand is brought about by inhibition of sympathetic nervous activity, whereas in the forearm it is the result of an active vasodilator mechanism (Edholm *et al.* 1956; Fox & Hilton, 1958). Further, the skin of the palmar surface of the hand contains abundant arteriovenous anastomoses, whereas they are few or absent in the forearm skin (Clara, 1956). This difference may be responsible for the greater increase in blood flow per unit volume of tissue, after intra-arterial histamine, in the hand compared with that in the forearm.

The response of certain smooth-muscle preparations to bradykinin is characterized by its slowness. The finding that the maximum vasodilatation in response to its intra-arterial injection occurred later than that in response to intra-arterial histamine or acetylcholine could reflect a similar slow action of bradykinin on the vessel wall.

However, the fact that the response to isoprenaline showed the same delay as bradykinin in reaching its maximum suggests that other factors may be responsible for this difference. With the exception of 5-hydroxytryptamine all the other drugs tested by intra-arterial injection produced roughly the same relative vasodilator effects in the skin of the forearm as in the whole forearm. This suggests the absence of differences in their actions on skin and muscle vessels. 5-hydroxytryptamine caused constriction in the skin but presumably vasodilatation in the muscle vessels, since the net effect on total forearm blood flow was a small increase. In contrast, Roddie, Shepherd & Whelan (1955) reported decreases in both hand blood flow and forearm blood flow, following an initial transient increase, with infusions of 5-hydroxytryptamine, and concluded that the drug acts by constricting the resistance vessels and dilating the vessels responsible for skin colour. The difference between this vasoconstrictor effect on total forearm blood flow found by Roddie *et al.* (1955) and the vasodilator effect found in the present experiments may be accounted for by two factors: first, the predominance with single injections of the initial transient vasodilatation in the muscle, and secondly, the difference in pre-injection skin blood flow levels—they were probably higher in the experiments of Roddie *et al.* and therefore the constrictor effect in the skin was more prominent. With the skin vessels largely constricted, the decrease in total forearm blood flow due to the vasoconstrictor action of 5-hydroxytryptamine on the skin would be relatively small, but the increase by the vasodilator effect on muscle vessels would be relatively enhanced.

Both bradykinin and histamine produced, on intravenous administration, vasodilatation in the face and neck at dose levels which were too low to produce effects on the forearm or hand. It is difficult to account for this

predilection although it has long been recognized that the face and neck are especially reactive (Lewis, 1927). It is in these regions that blushing in the young adult and flushing at the menopause are most marked, that the vasodilatation and sweating associated with strong gustatory stimuli are found, and, finally, they are among the few areas of the body in which the cold vasodilatation response is easily elicited (Fox & Wyatt, 1960). It has also been shown that the vasomotor control is different in adjacent areas of the face; in some the control is exercised by vasoconstrictor nerves, in others the control is predominantly by an active vasodilator mechanism (Fox, Goldsmith & Kidd, 1960*a*, *b*).

The greatly diminished vasodilator responses to injections of bradykinin and histamine, observed in one subject in the post-fainting state, points to the presence of some strong vasoconstrictor influence in this condition. The finding that the effect was most marked in the skin is in accord with the well known fact that after fainting the skin remains pale for a considerable period. There is strong evidence that posterior pituitary secretion is greatly increased during a faint and it has been suggested that the pallor of fainting results from the action of this hormone (Edholm, 1952). The observed resistance to the vasodilator action of bradykinin and histamine in this condition could readily be explained by the presence of a humoral agent rather than by the presence of a vasoconstrictor nerve effect, since both bradykinin and histamine dilate the vascular bed of the hand which is normally held in the vasoconstricted state by vasoconstrictor nerves. Furthermore, vasoconstrictor nerves do not seem to play a major role in controlling blood flow through the skin (Roddie, Shepherd & Whelan, 1957; Edholm, Fox & Macpherson, 1957) of the forearm.

Fox & Hilton (1958) pointed out that the association of sweating with a pale and apparently vasoconstricted skin in individuals in a state of shock was seemingly at variance with their hypothesis that bradykinin formed as a result of sweat-gland activity is responsible for the active vasodilatation in the forearm skin with indirect heating. They suggested that in an individual in a state of shock vasodilatation might be prevented by a stronger vasoconstrictor influence. The greatly diminished vasodilator responses to bradykinin and histamine encountered in the post-fainting state lends support to their suggestion.

## SUMMARY

1. The vasodilator activity of bradykinin in man has been compared with that of other vasoactive compounds given as single injections or infusions into the brachial artery or into the antecubital vein. Total forearm or hand blood flows were measured by venous occlusion plethysmo-

graphy and changes in skin blood flow were followed using surface calorimeters.

2. On intra-arterial administration both natural and synthetic bradykinin on a weight-for-weight basis were much more active than acetylcholine, although this is partly explained by the extremely rapid inactivation of acetylcholine in the blood stream. Bradykinin was about equiactive with isoprenaline in hand and forearm, but more active in the forearm and less active in the hand than histamine. On a molar basis, however, bradykinin was much more active than any other drug examined. It is concluded that when bradykinin is released in the human body its foremost action is vasodilatation.

3. While bradykinin, acetylcholine, isoprenaline and histamine caused only vasodilatation, 5-hydroxytryptamine produced a small increase in total forearm blood flow which was the resultant of a vasoconstrictor action on the skin blood flow and a vasodilator action on the muscle blood flow.

4. On intravenous administration the first effect of threshold doses of bradykinin (0·1–0·4 $\mu$g/kg) was a flushing of the face and neck. With larger doses this vasodilatation became intense and spread to the rest of the body. Histamine was more active than bradykinin by the intravenous route.

5. Some of the differences in the potency ratios between intra-arterial and intravenous injections may be explained by the different rates of inactivation of the drugs in the blood stream.

6. In one subject the effect of intra-arterial injection of bradykinin and histamine could be studied in the post-fainting state. The vasodilator responses were greatly reduced. It is concluded that this resistance to vasodilator substances results from the presence of a strong vasoconstrictor substance in the blood.

The authors are deeply indebted to the members of the 1960 Honours B.Sc. class in Physiology, and to the dental students at Guy's Hospital who were the subjects in some of these experiments. They also wish to thank Mr J. W. Jack for preparing the figures and Messrs Sandoz Ltd for kindly supplying the synthetic bradykinin.

## REFERENCES

Anrep, G. V., Barsoum, G. S. & Ibrahim, A. (1947). The histaminolytic action of blood during pregnancy. *J. Physiol.* **106**, 379–393.

Best, C. H. & McHenry, E. W. (1930). The inactivation of histamine. *J. Physiol.* **70**, 349–372.

Boissonnas, R. A., Guttmann, St, Jaquemond, P.-A., Konzett, H. & Stürmer, E. (1960). Synthesis and biological activity of peptides related to bradykinin. *Experientia*, **16**, 326–328.

Clara, M. (1956). *Die Arterio-venösen Anastomosen*. Wien: Springer-Verlag.

DUFF, P., GREENFIELD, A. D. M., SHEPHERD, J. T. & THOMPSON, I. D. (1953). A quantitative study of the response to acetylcholine and histamine of the blood vessels of the human hand and forearm. *J. Physiol.* **120**, 160–170.

EDHOLM, O. G. (1952). Physiological changes during fainting. In *Visceral Circulation*, ed. WOLSTENHOLME, G. E. W., pp. 256–270. A Ciba Foundation Symposium. London: Churchill.

EDHOLM, O. G., FOX, R. H. & MACPHERSON, R. K. (1956). The effect of body heating on the circulation in skin and muscle. *J. Physiol.* **134**, 612–619.

EDHOLM, O. G., FOX, R. H. & MACPHERSON, R. K. (1957). Vasomotor control of the cutaneous blood vessels in the human forearm. *J. Physiol.* **139**, 455–465.

ELLIOTT, D. F., HORTON, E. W. & LEWIS, G. P. (1960). Actions of pure bradykinin. *J. Physiol.* **153**, 473–480.

ELLIOTT, D. F., LEWIS, G. P. & HORTON, E. W. (1960*a*). The isolation of bradykinin: A plasma kinin from ox blood. *Biochem. J.* **74**, 15–16 *P*.

ELLIOTT, D. F., LEWIS, G. P. & HORTON, S. W. (1960*b*). The structure of bradykinin: A plasma kinin from ox blood. *Biochem. Biophys. res. Commun.* **3**, 87–91.

FOX, R. H., GOLDSMITH, R. & KIDD, D. J. (1960*a*). Cutaneous vasomotor nerves in the human ear and forehead. *J. Physiol.* **150**, 12–13 *P*.

FOX, R. H., GOLDSMITH, R. & KIDD, D. J. (1960*b*). The cutaneous vasomotor control in the human nose, lip and chin. *J. Physiol.* **150**, 22–23 *P*.

FOX, R. H. & HILTON, S. M. (1958). Bradykinin formation in human skin as a factor in heat vasodilatation. *J. Physiol.* **142**, 219–232.

FOX, R. H. & WYATT, H. T. (1960). The activity of the cold vasodilatation phenomenon in various body surface areas of man. *J. Physiol.* **151**, 30–31 *P*.

HATFIELD, H. S. (1950). A heat-flow meter. *J. Physiol.* **111**, 10–11 *P*.

HAWKINS, R. D. & MENDEL, B. (1947). Selective inhibition of pseudo-cholinesterase by diisopropyl fluorophosphonate. *Brit. J. Pharmacol.* **2**, 173–180.

HILTON, S. M. & LEWIS, G. P. (1955). The mechanism of the functional hyperaemia in the submandibular salivary gland. *J. Physiol.* **129**, 253–271.

HILTON, S. M. & LEWIS, G. P. (1956). The relationship between glandular activity, bradykinin formation and functional vasodilatation in the submandibular salivary gland. *J. Physiol.* **134**, 471–483.

HILTON, S. M. & LEWIS, G. P. (1957). Functional vasodilatation in the submandibular salivary gland. *Brit. Med. Bull.* **13**, 189–196.

LEWIS, G. P. (1960). Active polypeptides derived from plasma proteins. *Physiol. Rev.* **40**, 647–676.

LEWIS, T. (1927). *The Blood Vessels of the Human Skin and their Responses*, p. 258. London: Shaw and Sons.

ROCHA E SILVA, M., BERALDO, W. T. & ROSENFELD, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Amer. J. Physiol.* **156**, 261–273.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1955). The action of 5-hydroxytryptamine on the blood vessels of the human hand and forearm. *Brit. J. Pharmacol.* **10**, 445–450.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1957). The vasomotor nerve supply to the skin and muscle of the human forearm. *Clin. Sci.* **16**, 67–74.

*J. Physiol.* (1961), **157**, *pp.* 603–610
*With* 1 *text-figure*
*Printed in Great Britain*



# THE RESPONSE OF PIGLETS TO AMMONIUM CHLORIDE

BY N. HATEMI* AND R. A. McCANCE
*From the Department of Experimental Medicine, University of Cambridge*

(*Received* 15 *February* 1961)

The response of 'new-born' puppies and babies to ammonium chloride has been investigated (Cort & McCance, 1954; Hatemi & McCance, 1961; McCance & Hatemi, 1961) and the natural development of acid–base control after birth has been studied by Gordon, McNamara & Benjamin (1948), Widdowson & McCance (1958), Fomon, Harris & Jensen (1959) and McCance & Widdowson (1960). The general conclusion of these authors may be summarized by saying that the new-born animal makes a less effective response to acidifying drugs than an adult, whether the comparison is made on the basis of body weight or surface area, and there are clear indications that this may be clinically important (Darrow, da Silva & Stevenson, 1954; Hoffman, Parmelee & Grossman, 1948).

The pig has not yet been investigated and, since animals were available and pigs were known to differ in several ways from puppies and babies in their excretion of electrolytes, it was decided to make a short study of them.

## METHODS

Eight new-born piglets 1 or sometimes 2 days old (weighing 1·1–1·8 kg) were separated from the sow a few hours before the acidifying drug was to be given and placed in individual metabolism cages at 24 °C. The first specimen of urine was discarded and after timed specimens had been collected under toluol for about 2 hr, a sample of blood was taken from the heart and placed at once under paraffin oil. The sample was then centrifuged and the serum separated immediately. After the preliminary samples of urine and blood had been obtained, a solution of m/6-$NH_4Cl$ (25 ml. = 4·1 m-equiv/kg) warmed to 37° C was administered intraperitoneally to four piglets and by stomach tube to four others, and the time recorded. Per unit of body weight this dose was higher than the dose given to infants in the study of Hatemi & McCance (1961) and higher than the one given by Cort & McCance (1954) to new-born and adult dogs. Enough water was administered at two-hourly intervals by stomach tube to maintain a reasonable flow of urine and specimens were collected for about 8 hr and treated as described below. A second specimen of blood was taken 4 hr after the acidifying drug had been given.

Eight older piglets weighing 13–16 kg (10–12 weeks old) were investigated for comparative purposes. These animals had been weaned and had been taking a barley-maize-fishmeal-offal mixture (McCance, 1960). Each was placed in a metabolism cage at normal room temperature on the evening before the experiment was to begin and a preliminary collection of urine was made under toluol overnight, timed from the moment when the animal first emptied its bladder in the evening until it was first seen to empty its bladder in the morning.

* British Council Scholar.

When it had done this, pentobarbitone sodium 12 mg/kg body weight was administered intravenously. This kept the pigs lightly anaesthetized for 5–10 min. A sample of blood was taken from a vein in the leg or ear and M/6-$NH_4Cl$ solution at 37° C (25 ml. = 4·1 m-equiv/kg) was given intraperitoneally. Urine was collected under toluol in separately timed specimens for 8 hr and a second sample of blood was taken from the leg, again under very light pentobarbitone sodium anaesthesia, about 4 hr after the $NH_4Cl$ had been given. Urine specimens were removed as passed and kept in a refrigerator, and the specimens collected during the 8 hr after the acidifying salt had been administered were divided according to their volumes and times into four specimens, each representing a period of 2 hr. These were separately analysed, and so was the timed specimen passed before the drug was given.

The chemical methods were the same as those used by Hatemi & McCance (1961).

## RESULTS

Tables 1 and 2 show the changes in the volume and H-ion concentration of the urine before and after giving the $NH_4Cl$ and water to the new-born and older piglets. They also show the rates of excretion of titratable acid, ammonia, phosphates, chlorides, sodium and potassium, and, in the case of the older animals, creatinine. The average results for pH are shown in Fig. 1.

The administration of M/6-$NH_4Cl$ solution and water was followed by an increase in urine flow, but this increase was small in new-born animals. In older piglets the urine flow reached a level, after the administration of the drug, nearly three times that in the preliminary period. These results are in line with the observations of McCance & Wilkinson (1947), Ames (1953) and Adolph (1957) on the tendency of the newly born to excrete water less quickly and completely than older animals.

In the preliminary period the average $[H^+]$ in the urine was somewhat higher in the older piglets than it was in the new-born ones, although the difference was not statistically significant. After the administration of

TABLE 1. Urine volume, $[H^+] \times 10^{-7}$ and excretion rates of titratable acid, ammonia, phosphorus, chloride, sodium and potassium before and after giving $NH_4Cl$ to new-born piglets 1–2 days old. (Means and standard deviations are given)

| | Preliminary period | 0–2 hr | 2–4 hr | 4–6 hr | 6–8 hr |
|---|---|---|---|---|---|
| Urine flow (ml./min) | 0·21 ± 0·05 | 0·31 ± 0·08 | 0·29 ± 0·06 | 0·22 ± 0·04 | 0·17 ± 0·03 |
| $[H^+] \times 10^{-7}$ | 13·5 ± 21·0 | 3·84 ± 4·9 | 6·08 ± 7·9 | 24·2 + 22·0 | 52·2 ± 14·0 |
| Titratable acid (μequiv/kg/hr) | 24 ± 32 | 15 ± 10 | 19 ± 10 | 23 ± 13 | 28 ± 17 |
| Ammonia (μequiv/kg/hr) | 80 ± 30 | 127 ± 50 | 108 ± 48 | 102 ± 61 | 91 ± 37 |
| Phosphorus (mg/kg/hr) | 0·46 ± 0·52 | 0·38 ± 0·47 | 0·20 ± 0·24 | 0·39 ± 0·52 | 0·54 ± 0·53 |
| Chloride (μequiv/kg/hr) | 126 ± 62 | 179 ± 100 | 204 ± 115 | 226 ± 59 | 205 ± 65 |
| Sodium (μequiv/kg/hr) | 30 ± 21 | 28 ± 19 | 20 ± 17 | 29 ± 16 | 22 ± 13 |
| Potassium (μequiv/kg/hr) | 174 ± 56 | 205 ± 92 | 225 ± 90 | 228 ± 27 | 220 ± 15 |

TABLE 2. Urine volume, $[H^+] \times 10^{-7}$ and excretion rates of titratable acid, ammonia, phosphorus, creatinine, chloride, sodium and potassium before and after giving $NH_4Cl$ to piglets 10–12 weeks old. (Means and standard deviations are given)

| | Preliminary period | 0–2 hr | 2–4 hr | 4–6 hr | 6–8 hr |
|---|---|---|---|---|---|
| Urine flow (ml./min) | 0·34 ± 0·09 | 0·84 ± 0·21 | 1·25 ± 0·35 | 1·23 ± 0·39 | 0·73 ± 0·10 |
| $[H^+] \times 10^{-7}$ | 38·9 ± 21·0 | 10·3 ± 7·8 | 17·6 + 24 | 28·3 ± 6·9 | 44·6 ± 30·0 |
| Titratable acid (μequiv/kg/hr) | 68 ± 16 | 42 ± 13 | 43 ± 13 | 48 ± 19 | 70 ± 19 |
| Ammonia (μequiv/kg/hr) | 141 ± 27 | 201 ± 42 | 238 ± 45 | 235 ± 51 | 212 ± 38 |
| Phosphorus (mg/kg/hr) | 1·91 ± 0·47 | 1·27 ± 0·30 | 1·27 ± 0·46 | 1·39 ± 0·61 | 1·98 ± 0·57 |
| Creatinine (mg/kg/hr) | 0·73 ± 0·20 | 0·92 ± 0·71 | 0·88 ± 0·44 | 0·75 ± 0·25 | 0·82 ± 0·30 |
| Chloride (μequiv/kg/hr) | 63 ± 32 | 249 ± 105 | 365 ± 168 | 410 ± 157 | 312 ± 87 |
| Sodium (μequiv/kg/hr) | 19 ± 15 | 101 ± 70 | 167 ± 111 | 210 ± 142 | 170 ± 94 |
| Potassium (μequiv/kg/hr) | 32 ± 26 | 63 ± 25 | 103 ± 36 | 129 ± 37 | 111 ± 31 |

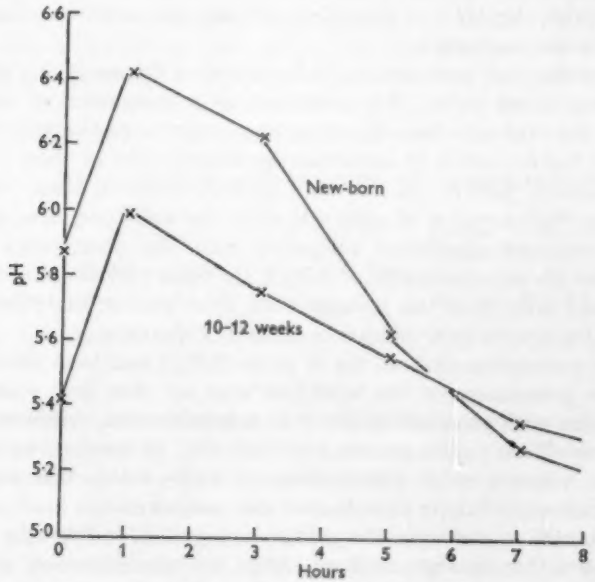



Fig. 1. The average pH of the urine before and after the administration of $NH_4Cl$ to young pigs.

$NH_4Cl$ solution, the $[H^+]$ of the urine fell in both groups of animals, and this fall was accompanied by the increase in urine flow. By the end of the experiment, the average pH of the urine was still above 5 at both ages.

Little inorganic phosphate was excreted by the new-born piglets and some of them excreted practically none. Towards the end of the experiments the amount of phosphate in the urine increased slightly, probably owing to starvation and the ensuing tissue breakdown (Widdowson & McCance, 1959). In contrast to this, older piglets had large amounts of phosphate in their urine, but after the administration of $NH_4Cl$ the rate of excretion always fell and, in some animals, to as little as 30–50 % of that in the preliminary period. This fall in the rate of excretion of phosphate was probably tubular in origin, for there was no corresponding fall in the excretion of endogenous creatinine.

In the preliminary period older piglets had a significantly higher average rate of excretion of titratable acid than new-born piglets, because their urines were more acid and also contained more phosphates. After the administration of M/6 $NH_4Cl$ solution the rate of titratable acid excretion fell in both groups. It began to rise only towards the end of the experiment, when the $[H^+]$ of the urine rose and the excretion of inorganic phosphorus also increased.

In the preliminary period and at all times after the acidifying drug had been given, older piglets had significantly higher rates of ammonia excretion than the new-born. Their average response to the acid load was rapid and the excretion of ammonia significantly higher than it was in the preliminary period. In new-born piglets, however, there was little increase in the excretion of ammonia after the acidifying drug and this increase was not significant compared with the preliminary period. Since, after the administration of $NH_4Cl$, the older piglets did not excrete a more acid urine than the younger ones, their greater excretion of ammonia did not seem to be related to the pH of the urine.

On the assumption that all the N in the $NH_4Cl$ had been converted to urea, the percentage of the hydrogen ions set free and excreted in combination with ammonia within 8 hr was calculated, and it was found that the new-born piglets excreted $5{\cdot}27 \pm 3{\cdot}56$ % of the hydrogen ions as ammonia, whereas older piglets excreted $13{\cdot}5 \pm 6{\cdot}2$ %, this percentage being significantly higher than that of the new-born.

The chloride excretion in the preliminary period was slightly lower in older piglets than in the new-born. After the administration of $NH_4Cl$, however, the rate increased by more than four times in older piglets, whereas in the new-born the increase was much smaller. Within 8 hr the older piglets excreted $50{\cdot}7 \pm 21{\cdot}2$ % of the chloride ions administered and the newborn piglets only $14{\cdot}7 \pm 2{\cdot}9$ %.

Before $NH_4Cl$ was given, the rate of excretion of sodium was nearly the same at both ages, and after the administration of the drug it remained practically unchanged in the new-born, whereas in older piglets it increased considerably. The excretion of potassium in the preliminary period was significantly higher in the new-born than in the older piglets. After the administration of $NH_4Cl$ the average rates of potassium excretion increased in both groups, but significantly more so in the new-born than in older piglets. New-born piglets therefore responded to an acidosis by increasing the rate of excretion of potassium without a significant change in that of sodium, whereas older piglets excreted both more sodium and more potassium.

These findings suggested that the concentrations of sodium in the serum of the new-born piglets might be low, and Table 3 shows that this in fact was true. Compared with the older animals the difference was statistically significant. The concentration of potassium in the serum, however, had a significantly higher average value in the new-born piglets. Before any drug was given, the average serum chloride concentration of the new-born piglets was somewhat lower than that of older piglets but the difference was not significant. The drug produced roughly the same effect on the serum chloride at both ages. New-born piglets tended to become cyanotic and partially asphyxiated while they were being held for heart puncture, and some of the results for total $CO_2$ were found to be unduly high. For this reason they have not been given in Table 3.

TABLE 3. Serum electrolytes in new-born piglets and in others 10–12 weeks old

| | Chloride (m-equiv/l.) | | Total $CO_2$ (m-equiv/l.) | | Sodium (m-equiv/l.) | Potassium (m-equiv/l.) | Phosphorus (mg/100 ml.) |
|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | | | |
| New-born piglets | 99 ± 2·7 | 105 ± 3·6 | — | — | 125 ± 4·8 | 5·5 ± 0·3 | 11·2 ± 2·2 |
| Piglets 10–12 weeks old | 104 ± 3·2 | 108 ± 2·7 | 28 ± 2·9 | 24 ± 2·2 | 140 ± 5·0 | 4·8 ± 0·6 | 10·4 ± 1·7 |

## DISCUSSION

These results have revealed some peculiarities in the response of young pigs to an ammonium chloride acidosis and also demonstrated some developmental aspects of acid–base control.

It has been shown before that the kidneys of new-born infants may not lower the pH of the urine so rapidly or completely as those of adults (McCance & Hatemi, 1961). Piglets in these experiments gave a poorer response than new-born infants, but the results were complicated to some extent by the diuresis (Cort & McCance, 1954), although this was relatively small in the newly born animals.

The excretion of titratable acid is closely related to the amount of

phosphate in mammalian urine and urates in the urine of birds (Wolbach, 1955), and the minimum pH attainable (Schiess, Ayer, Lotspeich & Pitts, 1948; Wrong & Davies, 1959). New-born piglets were limited in both respects, since they excreted very little phosphate and they did not lower the pH of the urine for some hours. Although older piglets excreted much larger amounts of phosphate in their urines initially, their response was little better because the pH of their urine also rose and, furthermore, the excretion of phosphate decreased after the administration of $NH_4Cl$. As the older piglets were given small amounts of anaesthetic before the blood was taken, the decrease in phosphate excretion may have been due to this (de Wardener, 1958), but the rate of endogenous creatinine excretion did not suggest any decrease in glomerular filtration rate. The possibility of diurnal rhythm of phosphate excretion was also ruled out, since in control animals given no acidifying drug phosphate excretion did not fall during the day. The response of piglets 10–12 weeks old to an acute acidosis was therefore unlike that of adult dogs, which trebled the rate of excretion of phosphate after being given $NH_4Cl$ or $(NH_4)_2SO_4$ (Cort & McCance, 1954). It has been shown in man that the administration of phosphates makes no difference to the excretion of total base after giving ammonium chloride but it decreases the excretion of chlorides (Hatemi & McCance, 1961). It is suggested that in all species it is the output of cations which regulates the quantity of anions excreted in the urine after the administration of ammonium chloride. Owing to species differences in tubular function, phosphates are preferentially excreted in the dog and to a lesser extent in man, but this is not so in the pig, in which animal chlorides follow the sodium into the urine to such an extent that the excretion of phosphate actually falls.

Cort & McCance (1954) found that the excretion of ammonia by puppies in response to an acidosis had greatly improved by the time they were 15 days of age. The present study demonstrates that in pigs this was also the case between birth and by 10–12 weeks of age. This improvement may be due to increased intracellular production of ammonia (Davies & Yudkin, 1952; Hines & McCance, 1954; Orloff, 1956), since the average values for urinary pH after the administration of ammonium chloride were roughly the same at each age.

New-born piglets are known to excrete Na less freely than new-born puppies (McCance & Widdowson, 1958). Without free excretion of potassium this must limit their output of administered anions, and new-born piglets in the present investigation excreted only 14% of the chloride in the $NH_4Cl$, whereas puppies studied by Cort & McCance (1954) excreted 47% within 6 hr. Largely owing to the increased excretion of sodium the older piglets excreted 50% of the chloride ions within 8 hr.

The effect of development on the composition of the serum and extracellular fluids has been studied by Widdowson & McCance (1956) in man and pig. These authors reported significantly lower levels of serum sodium in pig foetuses 46 days old ($122 \pm 9{\cdot}9$ m-equiv/l.) than in piglets at birth ($141 \pm 4{\cdot}8$ m-equiv/l.). Serum potassium values, however, were $17{\cdot}5 \pm 2{\cdot}4$ m-equiv/l. in foetal sera and $8{\cdot}6 \pm 2{\cdot}6$ m-equiv/l. at birth. In the present study, the average concentration of sodium and chloride in the serum of the piglets 24–48 h after birth was near to that reported for the foetuses in 1956. 'Normal' serum sodium concentrations have, however, also been found in piglets aged 24–48 h and the cause of the low values in piglets from some litters is obscure. The problem will be further investigated. The average serum sodium concentration in older piglets was nearly equal to that reported at birth by Widdowson & McCance (1956). The average values for serum potassium in both the younger and older piglets were lower than the average value given for piglets at birth by Widdowson & McCance (1956).

The serum electrolyte concentrations found in the present investigation may help to explain the electrolyte excretions in the two age groups. Experimental work in man (Schwartz, Jensen & Relman, 1954; Clarke, Evans, MacIntyre & Milne, 1955) has demonstrated that the excretion of sodium during an acidosis increases very little in sodium-depleted subjects, who excrete instead larger amounts of potassium than normal subjects. In new-born piglets no increase in sodium excretion was noted after $NH_4Cl$. Clarke *et al.* (1955) reported a very small increase in salt-deficient men, but the serum sodium levels in their subjects were not as low as those now found in new-born piglets. There is, furthermore, an inherent difference between the new-born and the adult animals in their readiness to excrete sodium. Older piglets which had normal serum sodium and potassium concentrations, and probably a relatively adult pattern of metabolism, responded by increasing both sodium and potassium excretion after the administration of the acidifying drug.

SUMMARY

1. $NH_4Cl$ was administered to piglets 1–2 days old and to animals 10–12 weeks old weighing about 15 kg.

2. In both groups the average pH of the urine rose and did not regain its initial level for 5–6 hr.

3. In new-born animals the excretion of phosphates was at all times negligible and that of $NH_3$ did not increase significantly after giving $NH_4Cl$.

4. In older animals the excretion of phosphates fell after $NH_4Cl$ but that of $NH_3$ rose significantly.

5. In new-born animals $NH_4Cl$ increased the excretion of potassium but not sodium. In older animals the excretion of both cations was increased.

## REFERENCES


ADOLPH, E. F. (1957). Ontogeny of physiological regulations in the rat. *Quart. Rev. Biol.* **32**, 89–137.

AMES, R. D. (1953). Urinary water excretion and neurohypophysial function in full-term and premature infants shortly after birth. *Pediatrics*, **12**, 272–282.

CLARKE, E., EVANS, B. M., MACINTYRE, I. & MILNE, M. D. (1955). Acidosis in experimental electrolyte depletion. *Clin. Sci.* **14**, 421–440.

CORT, J. H. & MCCANCE, R. A. (1954). The renal response of puppies to an acidosis. *J. Physiol* **124**, 358–369.

DARROW, D. C., DA SILVA, M. M. & STEVENSON, S. S. (1945). Production of acidosis in premature infants by protein milk. *J. Pediat.* **27**, 43–58.

DAVIES, B. M. A. & YUDKIN, J. (1952). Studies in biochemical adaptation. The origin of urinary ammonia as indicated by the effect of chronic acidosis and alkalosis on some renal enzymes in the rat. *Biochem. J.* **52**, 407–412.

DE WARDENER, H. (1958). *The Kidney*. London: J. and A. Churchill Ltd.

FOMON, S. J., HARRIS, D. M. & JENSEN, R. L. (1959). Acidification of the urine by infants fed human milk and whole cow's milk. *Pediatrics*, **23**, 113–120.

GORDON, H. H., MCNAMARA, H. & BENJAMIN, H. R. (1948). The response of young infants to ingestion of ammonium chloride. *Pediatrics*, **2**, 290–302.

HATEMI, N. & MCCANCE, R. A. (1961). Renal aspects of acid-base control in the newly born. III. Response to acidifying drugs. *Acta paediat., Stockh.* (in the Press).

HINES, B. E. & MCCANCE, R. A. (1954). Ammonia formation from glutamine by kidney slices from adult and newborn animals. *J. Physiol.* **124**, 8–61.

HOFFMAN, W. S., PARMELEE, A. H. & GROSSMAN, A. (1948). Mechanism of production of acidosis in premature infants by protein milk. *Amer. J. Dis. Child.* **75**, 637–659.

MCCANCE, R. A. (1960). Severe undernutrition in growing and adult animals. I. Production and general effects. *Brit. J. Nutr.* **14**, 59–73.

MCCANCE, R. A. & HATEMI, N. (1961). Control of acid-base stability in the newly born. *Lancet*, **280**, 293–297.

MCCANCE, R. A. & WIDDOWSON, E. M. (1958). The response of the newborn puppy to water, salt and food. *J. Physiol.* **141**, 81–87.

MCCANCE, R. A. & WIDDOWSON, E. M. (1960). Renal aspects of acid-base control in the newly born. I. Natural development. *Acta paediat., Stockh.*, **49**, 409–414.

MCCANCE, R. A. & WILKINSON, E. (1947). The response of adult and suckling rats to administration of water and hypertonic solution of urea and salt. *J. Physiol.* **106**, 256–263.

ORLOFF, J. (1956). The role of the kidney in the regulation of acid-base balance. *Yale J. Biol. Med.* **29**, 211–228.

SCHIESS, W. A., AYER, J. L., LOTSPEICH, W. D. & PITTS, R. F. (1948). The renal regulation of acid-base balance in man. II. Factors affecting the excretion of titratable acid by the normal human subject. *J. clin. Invest.* **27**, 57–64.

SCHWARTZ, W. B., JENSEN, R. L. & RELMAN, A. S. (1954). The disposition of acid administered to sodium depleted subjects: the renal response and the role of the whole body buffers. *J. clin. Invest.* **33**, 587–597.

WIDDOWSON, E. M. & MCCANCE, R. A. (1956). The effect of development on the composition of the serum and extracellular fluids. *Clin. Sci.* **15**, 361–365.

WIDDOWSON, E. M. & MCCANCE, R. A. (1958). The development of acid base control. *Ciba Foundation Colloquia on Ageing*, **4**, 209–219.

WIDDOWSON, E. M. & MCCANCE, R. A. (1959). The effect of food and growth on the metabolism of phosphorus in the newly born. *Acta paediat., Stockh.*, **48**, 383–387.

WOLBACH, R. A. (1955). Renal regulation of acid base balance in the chicken. *Amer. J. Physiol.* **181**, 149–156.

WRONG, I. & DAVIES, H. E. F. (1959). The excretion of acid in renal disease. *Quart. J. Med.* (N.S.), **28**, 259–313.

*J. Physiol.* (1961), **157**, *pp.* 611–622
*With 6 text-figures*
*Printed in Great Britain*



# THE MEASUREMENT OF BRONCHOCONSTRICTION *IN VIVO*

BY J. L. D'SILVA AND A. F. LEWIS*

*From the Department of Physiology,*
*King's College, London, W.C.2*

(*Received* 15 *March* 1961)

## INTRODUCTION

It is more than a century since constriction of the lower respiratory tract by its intrinsic musculature was demonstrated. Dixon & Brodie (1903) recognized that bronchial constriction could be associated with changes in lung distensibility, but their experiments did not show the latter change. Variations of their technique have since been described, for example, the method of Konzett & Rössler (1940). All these methods presented a composite picture of changing lung distensibility and flow-resistance. Because of these methods and their ready application to pharmacological problems there emerged the new term 'pneumo-constriction' (Delaunois, Dautrebande & Heymans, 1956), which embraces in its meaning changes of airway resistance and changes of elastance due to closure of alveolar units.

In 1939 Bayliss & Robertson described a method of measuring the viscous and elastic properties of the lung concurrently, and in absolute terms. This work has since expanded into a wide field of pulmonary mechanics.

The aim of the present work is to calculate the mean bronchiolar calibre from concurrent measurements of elastance and flow-resistance.

## THEORY

The elastic properties of a system, such as the lungs, are described by the term *elastance* which is the ratio of impressed pressure change to volume change. The term *compliance* is the reciprocal of this value and is the equivalent of the electrical term *capacitance*. The viscous or flow-resistive properties are divided into those due to air flow (airway resistance) and those due to tissue movement (tissue resistance).

The airway is visualized in the form of a greatly simplified model. The upper part consists of comparatively rigid tubes of constant calibre under the conditions of experiment in a thoracotomized animal. This opens into a group of tubes in parallel which represent the lower airway. These latter

* Present address: Department of Physiology, Charing Cross Hospital Medical School, London, W.C.2.

tubes are of equal radius, they lack structural rigidity, and have sufficient muscle to allow of considerable variation in calibre. Each tube opens into an alveolar space, all the spaces being of equal capacity. The tube and alveolar space are referred to later as an alveolar unit.

The following assumptions have been made with regard to the physiological properties of the system: (*a*) that tissue elasticity remains constant under physiological conditions, (*b*) that changes in the elastance of the lung are due to variations in the number of lower airway units open and participating in ventilation, (*c*) that changes in resistance to gas flow are due to changes in calibre of the lower airway tubes together with variations in the geometry of the system due to closure of units, (*d*) lung inertia is very small at low frequencies (DuBois, 1953) and may be neglected, and (*e*) lung tissue resistance is small and unlikely to alter (Brody & DuBois, 1956).

Let the total resistance of the whole system be $R$, and let $R_a$ represent that part of the viscous resistance that is unlikely to change during bronchoconstriction. It therefore includes the airway resistance of the upper airway together with the tissue resistance of the whole lung. Let the resistance of each tube in the lower airway be $R_b$, and let the total number of tubes in parallel be $n$. Then,

$$\frac{1}{R-R_a} = \frac{n}{R_b},$$

or

$$R = R_a + \frac{R_b}{n}. \tag{1}$$

If the total compliance of the system is $C$ and the compliance of each alveolar sac is $c$, then

$$C = nc = \frac{1}{K},$$

where $K$ is the elastance of the whole lung.

The subscript $o$ indicates the resting state, and $C_o$, $K_o$ and $R_o$ are defined as equal to unity; that is, changes in each of these parameters are calculated in terms of their respective values in the resting state. The subscript $t$ indicates the state at any instant during active bronchoconstriction.

Since

$$\frac{C_t}{C_o} = \frac{n_t c}{n_o c},$$

$$C_t = \frac{n_t}{n_o}, \quad \text{as} \quad C_o = 1$$

and

$$K_t = \frac{n_o}{n_t}.$$

Equation (1), at time $t$, can be written

$$R_t = R_{at} + \frac{R_{bt}}{n_t}. \tag{2}$$

As $n_t$ can be expressed in terms of $K_t$ and, by definition, $R_{at}$ does not differ from $R_{ao}$, then substituting in equation (2):

$$R_t = R_{ao} + R_{bt}\frac{K_t}{n_o},$$

whence

$$R_{bt} = \frac{n_o}{K_t}(R_t - R_{ao}). \tag{3}$$

For the streamlined flow of gas through a tube, the resistance varies inversely with the fourth power of the radius. If the resting radius, $r_{bo}$, of each tube in the lower airway is defined as unity, then

$$\frac{R_{bt}}{R_{bo}} = \frac{r^4_{bo}}{r^4_{bt}}$$

and so

$$R_{bt} = \frac{R_{bo}}{r^4_{bt}} \quad \text{as} \quad r^4_{bo} = 1.$$

Substituting in equation (3) for $R_{bt}$:

$$\frac{R_{bo}}{r^4_b} = \frac{n_o}{K_t}(R_t - R_{ao}).$$

Therefore,

$$r^4_{bt} = \frac{K_t}{R_t - R_{ao}} \cdot \frac{R_{bo}}{n_o}.$$

But, from (1), written for the resting state:

$$\frac{R_{bo}}{n_o} = R_o - R_{ao}.$$

Therefore,

$$r^4_{bt} = K_t \cdot \frac{R_o - R_{ao}}{R_t - R_{ao}}. \tag{4}$$

In the model on which the above calculations are based each lower airway unit is associated with one alveolar unit so that there is a linear relationship between elastance and flow resistance. If this simplified model closely represents the lung, there should also be a linear relationship between lung elastance and flow resistance.

## METHODS

Guinea-pigs, rabbits and kittens between 0·3 and 0·8 kg were anaesthetized with an intraperitoneal injection of Nembutal (40 mg/kg). The trachea was cannulated and the animal was connected to a miniature Palmer 'Ideal' respiration pump. Constant volume artificial respiration was given, allowing passive expiration, at a frequency which was just sufficient

to suppress spontaneous respiration. The peak inflation pressure was adjusted to 12 cm water so that the tidal volume was equal to about half vital capacity.

Expiration was limited by a water valve set at 2 cm water to prevent complete collapse of the lungs when the chest was opened. The internal mammary arteries were ligated and the anterior chest wall was widely resected. The diaphragm was retracted and branches of the phrenic nerve were cut near the diaphragm. The lungs were covered with a thin layer of gauze moistened with normal saline, care being taken not to compress any part of them. Tracheal pressures were monitored by an aneroid barometer and this also provided evidence of bronchoconstriction.

Fig. 1. A typical pressure–volume diagram for the lung. The points $A$, $B$, $C$, and $D$ appear successively on the $Y$ axis and denote the tracheal pressures at the instants of zero inflation, half inflation, full inflation and half deflation respectively. The volume change is shown by the horizontal line below the diagram. The pressures at $B$ and $D$ occur at the mid point of the volume change. $AC$ = elastic pressure ($P_E$); $BD$ = resistive pressure ($P_R$).

Another pump, delivering a sinusoidal output on a closed circuit, was used for the measurement of the dynamic pressure–volume relationships of the lungs. The pump stroke and frequency were fixed and a sliding potentiometer, moving synchronously with the piston of the pump, gave an output potential proportional to the displacement of the piston for display on the X axis of an oscilloscope.

Tracheal pressure was measured by a capacitance manometer which formed one arm of a capacitance bridge. The output of the bridge, suitably rectified and amplified, was displayed on the Y axis of the oscilloscope.

Contacts placed at 90° intervals on the final drive shaft of the pump motor indicated directly on the oscilloscope the tracheal pressures at the extremes of displacement and at mid-displacement of the sinusoidal pump. Figure 1 shows a typical pressure–volume outline. The interval between the two points indicating the extremes of displacement gives the elastic pressure ($P_E$) at full inflation, while the interval between the two points at mid-displacement gives the resistive pressure drop ($P_R$) during maximal air flow.

During artificial ventilation various methods were used to induce bronchoconstriction. Histamine was given by aerosol and methacholine by aerosol or by intramuscular injection. Aerosols were generated by a Wright nebulizer at an air pressure of 12 lb./sq.in. (0·84 kg/cm²). Dosage was related to the time of exposure and the concentration of the solution. Multi-channel valves were used for rapid change over from one nebulizer to another.

The course of the lung changes was followed by switching the animal to the sinusoidal pump at regular intervals of 30 or 60 sec. A multi-channel valve was used in the change over, so that the lungs were always set at 2 cm water. The outlines obtained by display of the two out-of-phase potentials on the two axes of the oscilloscope were ellipses after allowance had been made for the very slight non-linearity of the capacitance manometer. Some gas absorption occurred during the first two cycles of the sinusoidal pump, but stability was usually reached by the third cycle, which gave a closed outline, so that the initial and final zero-displacement marks were super-imposed, as in Fig. 1.

The ellipses were photographed and the results read from the film directly by projection on a suitably calibrated scale. Thus simultaneous elastance and resistance measurements were obtained at known intervals throughout the experiment.

In other experiments the relationship between resistance and elastance in atropinized animals was determined after successive incremental increases of the inflation-volume from the artificial-respiration pump. Thus the number of alveolar units in the system was increased progressively. After increasing tidal volume the peak tracheal pressure rose, and then fell and stability was reached after 2–3 min. Once opened, the rate of closure of the newly opened units was very slow.

## RESULTS

### *Relationship between resistance to air flow and elastance in atropinized animals*

When the tidal volume of positive-pressure ventilation in an animal is increased, the pressure generated opens additional alveolar units. Hence equation (1) can be written as follows:

In the resting state

$$R_o = R_{ao} + \frac{R_{bo}}{n_o};$$

during increased ventilation,

$$R_t = R_{ao} + \frac{R_{bo}}{n_t}.$$

Since

$$n_t = \frac{n_o}{K_t}, \quad R_t = R_{ao} + \frac{R_{bo}}{n_o} . K_t .$$

Figure 2 shows the results of representative experiments in each of the three species used. The relationship between elastance and resistance to air flow was linear, low values of elastance and resistance being obtained

when the tidal volume was high, because more alveolar units were being ventilated. When artificial ventilation was carried out at a tidal volume of about 28 ml./kg body weight, a minimum value was obtained for elastance.

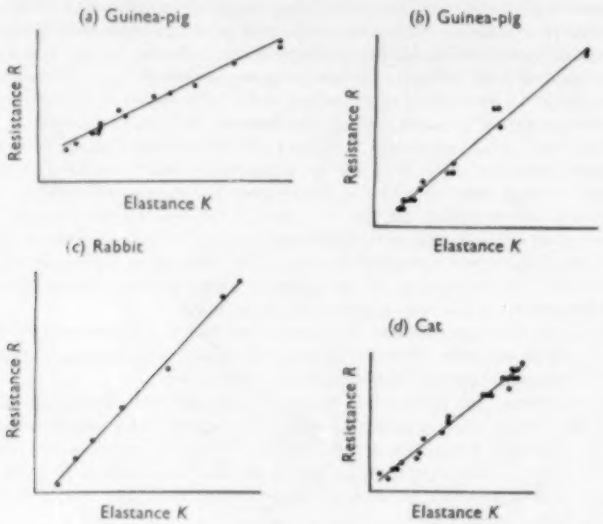



Fig. 2. Graphs showing the relationship between elastance and resistance in three species as the lung inflation volume was progressively increased. All the animals were atropinized.

### *The effect of histamine and methacholine on elastance and resistance to air flow*

Figure 3 shows the changes in elastance and total resistance to air flow in response to methacholine injected intramuscularly. Values of $K_t$ and $R_t$ are expressed as multiples of the resting elastance $K_o$ and resistance $R_o$ at the start of the experiment. In this experiment elastance has increased twofold and resistance nearly tenfold. The lowest curve represents the lower airway calibre $r_{bt}$ calculated from equation (4). This is also expressed as a fraction of the resting value $r_{bo}$.

This experiment alone does not provide a value for $R_{ao}$, but it is possible to calculate this from the equation to the linear plots shown in Fig. 2. For technical reasons it was not possible to obtain a value for $R_{ao}$ in all experiments. The three curves in Fig. 4 have been calculated from equation (4) on the assumption that $R_{ao}$ contributes 0·2, 0·5, and 0·7 of the total flow-resistance, $R_o$. All the experimentally determined values lie within this range. These curves, relating total resistance to radius, are not

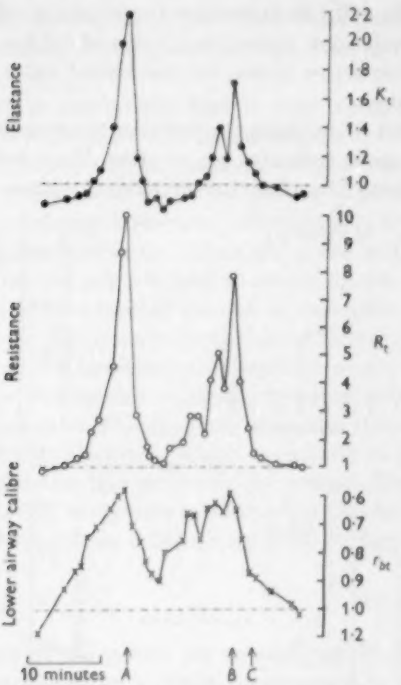



**Fig. 3.** Simultaneous values for elastance and resistance obtained by intramuscular injection of methacholine chloride. The lowest curve shows the calculated change in lower airway calibre. Adrenaline was injected intravenously at *A* and *B*, and atropine at *C*.

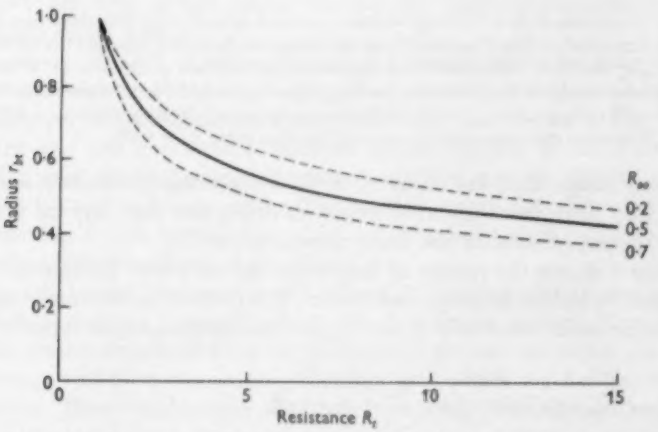



**Fig. 4.** Values for lower airway radius $r_{bt}$ calculated by using different values for the constant-resistance component $R_{ao}$.

very different whether $R_{ao}$ contributes 0·2 or 0·7 of the total flow-resistance. In subsequent calculations, therefore, a value of 0·5 has been assumed for $R_{ao}$ and, within the above limits, the calculated value of $r_{bt}$ is accurate within 12%.

If the only effect of inducing bronchiolar constriction was to impose a progressively greater resistance to air flow, there would have been no change in elastance. The observation that elastance has considerably

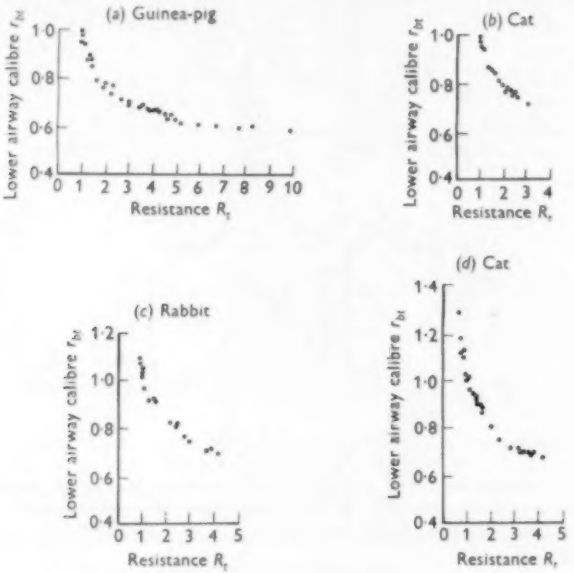



Fig. 5. Calculated lower airway calibre $r_{bt}$ plotted against total flow-resistance in four experiments. The drugs used to secure changes in bronchial tone and the values of $r_{bt}$ in the limit when resistance approaches infinity are as follows: (*a*) Intramuscular methacholine chloride, limiting value $r_{bt} = 0{\cdot}57$. (*b*) Methacholine by aerosol, limiting value $r_{bt} = 0{\cdot}65$. (*c*) Histamine by aerosol, limiting value $r_{bt} = 0{\cdot}65$. (*d*) Histamine and adrenaline by aerosol, limiting value $r_{bt} = 0{\cdot}63$.

increased means that the capacity of the system has diminished and this could only have come about if tissue elasticity did not vary as a result of the closure of some of the lower airway units.

Figure 5 shows the results of four experiments where various methods were used to induce bronchoconstriction. The plots of lower airway calibre, $r_{bt}$, against total resistance $R_t$ are hyperbolae of the general form

$$r_{bt} = \frac{R_t}{a + b.R_t}.$$

When $R_t \rightarrow$ infinity, $r_{bt}$ is equal to $1/b$. This is the value of $r_{bt}$ which would ensure complete closure of the lower airway. This is the critical closing calibre.

In the guinea-pig it was usually easy to take constriction to the mean critical closing calibre. At this point measurements became difficult as the changes were very rapid and of considerable dimensions. Figure 5*a* is from the same experiment as Fig. 3 and shows a plateau (gradient less than 1/100) at $r_{bt} = 0{\cdot}6$ approximately. The relatively few observations at this degree of bronchoconstriction reflect the speed and instability of the changes taking place and not difficulty in obtaining the changes. In some experiments a greater than tenfold increase in resistance was achieved. In the kitten and rabbit the mean critical closing calibre of the bronchioles was not reached, as the large doses of bronchoconstrictor drugs needed affected the heart. The results obtained, however, when the resistance increased fourfold or more, fell on curves of similar shape, so that calculations were made of the values of $r_{bt}$ which were likely to have been reached if bronchiolar constriction had proceeded far enough. The values obtained were again near to 0·6, which means that when the bronchioles contract to about 60 % of their normal calibre, they close suddenly and completely.

## DISCUSSION

The susceptibility of the guinea-pig to asphyxia during bronchospasm has long been assumed to be a direct consequence of the large mass of circular muscle around the bronchioles (Macklin, 1929). There is no doubt that this muscle can exert sufficient tension to constrict the lower airways to their critical closing diameter. After this critical diameter has been reached, however, *in vivo* studies will not provide more information about the bronchiolar muscle.

Martin & Proctor (1958) constructed pressure–volume curves for the isolated trachea and bronchi of a dog. Their experimental figures have been converted into tension–radius values, and these are shown in Fig. 6. The trachea and the two sizes of bronchi studied appear to have an outer limiting coat which limits enlargement, so that tension rapidly rises to infinity beyond a certain radius. The trachea shows a similar trend upon constriction and it seems likely that a structurally rigid inner coat prevented complete collapse of the tube.

The two bronchi behaved quite differently on constriction and rapidly reached a state of instability from which collapse ensued. This critical radius can be estimated from the inflexion of the curves and is about 0·4 of resting value for the larger bronchus and about 0·7 for the smaller bronchus. These values were obtained from fluid-filled airways and they

are therefore smaller than would be expected *in vivo*, where a fluid interface exists and facilitates closure.

Similar figures are not available for the bronchioles but it is reasonable to suggest that the trend from trachea to small bronchus should continue. It appears, therefore, that all the airways have an upper limit of expansion due to an outer limiting coat but that the smaller tubes are not sufficiently

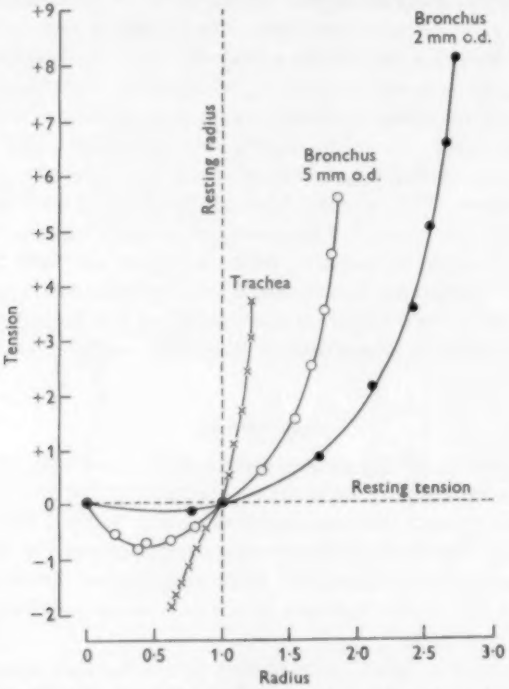



Fig. 6. Calculated tension–radius curves for three sizes of respiratory airway prepared from values for pressure and volume measured *in vitro* by Martin & Proctor (1958). Tension is expressed in arbitrary units and radius is given as a fraction of the resting value. The points of inflexion of the curves for the two smaller tubes can only be estimated as there are insufficient measurements in this region.

rigid to prevent their collapse on constriction. In addition, the smaller airways are more distensible than the larger and presumably transmural pressures are predominant in determining their normal resting diameter in the intact animal.

The problem of transmural bronchiolar pressures in the intact animal is complex. In the thoracotomized animal bronchiolar diameter depends upon the balance between its muscle tension, structural rigidity, and the

surface tension of its secretions. By Laplace's law the centripetal force due to fluid surface tension increases inversely as the radius. A point will then be reached during bronchiolar constriction when this force equals and then exceeds the structural rigidity of the tube. At this point of unstable equilibrium collapse commences and further active constriction will only make collapse more certain. In the thoracotomized animal therefore the actual diameter is critical in the absence of externally applied transmural pressures. For this reason the index of critical closing *diameter* is preferred in this work to that of *pressure*.

A collapsed tube will only expand from the application of an externally directed transmural pressure. There is no evidence that re-establishment of the airway takes place spontaneously when muscular contraction is relaxed. Alveolar units opened by over-inflation stay open for a considerable time after the over-inflation is discontinued—thus the efficacy of a yawn. The closure of the newly opened units seems to follow an exponential law and an hour or more may pass before elastance returns to the original reading. The presence of increased bronchial secretions considerably hastens this return.

It has been shown that asthmatic patients are more sensitive to inhaled histamine or acetylcholine than normal subjects, but there is no evidence that sensitized animals behave similarly, and it is suggested that the concept of critical closure may explain the results in man. Curry (1946, 1947) measured the vital capacity of asthmatic subjects and showed that this was reduced by doses of histamine that were without effect on the vital capacity of normal subjects. A change of vital capacity is not an indication of altered airway resistance *per se* and presumably the change represents increased elastance due to closure of alveolar units. Radford & Lefcoe (1955) quote unpublished observations by Mead that lung air-flow resistance could be increased three or fourfold in normal subjects by histamine aerosol without concurrent increase in elastance. However, two asthmatic subjects showed increases of elastance of 1·4–2 times during asthmatic attacks of moderate severity. This may have been due to a proportion of the bronchioles reaching their critical closing calibre, the process being assisted by increased amounts of bronchial secretion.

SUMMARY

1. Values of elastance and flow resistance determined simultaneously during bronchoconstriction are shown, on theoretical grounds, to provide an index of lower airway calibre.

2. Experiments are described to support this interpretation and to determine this index.

3. The significance of the changes is discussed with reference to the influence of surface tension upon critical closing diameter.

## REFERENCES


BAYLISS, L. E. & ROBERTSON, G. W. (1939). The visco-elastic properties of the lungs. *Quart. J. exp. Physiol.* **29**, 27–47.

BRODY, A. W. & DUBOIS, A. B. (1956). Determination of tissue, airway and total resistance to respiration in cats. *J. appl. Physiol.* **9**, 213–218.

CURRY, J. J. (1946). The action of histamine on the respiratory tract in normal and asthmatic subjects. *J. clin. Invest.* **25**, 785–791.

CURRY, J. J. (1947). Comparative action of acetyl-beta-methyl choline and histamine on the respiratory tract in normals, patients with hay fever, and subjects with bronchial asthma. *J. clin. Invest.* **26**, 430–438.

DELAUNOIS, A. L., DAUTREBANDE, L. & HEYMANS, C. (1956). Method for administering micromicellar aerosols to guinea-pig isolated lungs. *Arch. int. Pharmacodyn.* **108**, 238–251.

DIXON, W. E. & BRODIE, T. G. (1903). Contributions to the physiology of the lungs. Part I. The bronchial muscles, their innervation, and the action of drugs upon them. *J. Physiol.* **29**, 97–173.

DUBOIS, A. B. (1953). Resistance to breathing measured by driving the chest at 6 c.p.s. *Fed. Proc.* **12**, 35–36.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. exp. Path. Pharmak.* **195**, 71–74.

MACKLIN, C. C. (1929). The musculature of the bronchi and lungs. *Physiol. Rev.* **9**, 1–60.

MARTIN, H. B. & PROCTOR, D. F. (1958). Pressure-volume measurements on dog bronchi. *J. appl. Physiol.* **13**, 337–343.

RADFORD, E. P. & LEFCOE, N. M. (1955). Effects of bronchoconstriction on elastic properties of excised lungs and bronchi. *Amer. J. Physiol.* **180**, 479–484.

THE JOURNAL OF PHYSIOLOGY, AUGUST 1961

VOL. 157, NO. 3

## CONTENTS

PAGE

LAMB, J. F. THE CHLORIDE CONTENT OF RAT AURICLE . . . . . 415

CARO, C. G. AND McDONALD, D. A. THE RELATION OF PULSATILE PRESSURE AND FLOW IN THE PULMONARY VASCULAR BED. . . . . 426

DE LIMA, T. AND LOCKETT, MARY F. THE ACTION OF CHLOROTHIAZIDE IN THE PERFUSED CAT KIDNEY . . . . . 454

ALLISON, P. R., DALY, I. DE BURGH AND WAALER, B. A. BRONCHIAL CIRCULATION AND PULMONARY VASOMOTOR NERVE RESPONSES IN ISOLATED PERFUSED LUNGS. . . . . 462

WAALER, B. A. THE EFFECT OF BRADYKININ IN AN ISOLATED PERFUSED DOG LUNG PREPARATION . . . . . 475

ECCLES, ROSAMOND M. AND LIBET, B. ORIGIN AND BLOCKADE OF THE SYNAPTIC RESPONSES OF CURARIZED SYMPATHETIC GANGLIA . . . . 484

DOSEKUN, F. O. THE MEASUREMENT OF METABOLIC AND VASCULAR RESPONSES IN THE THYROID GLAND WITH OBSERVATIONS ON ITS RESPONSES TO INSULIN, GLUCOSE AND ADRENALINE . . . . . 504

BARKER, D. AND GIDUMAL, JANKI L. THE MORPHOLOGY OF INTRAFUSAL MUSCLE FIBRES IN THE CAT . . . . . 513

MYANT, N. B. AND OSORIO, C. COMPETITION FOR TRIIODOTHYRONINE BETWEEN SERUM PROTEINS AND RED CELLS . . . . . 529

FLETCHER, K. AND MYANT, N. B. PARTIAL REVERSAL OF THE EFFECTS OF THYROXINE ON LIPID SYNTHESIS IN RAT LIVER BY THE ADDITION OF COFACTORS *IN VITRO* . . . . . 542

NATHAN, P. W. AND SEARS, T. A. SOME FACTORS CONCERNED IN DIFFERENTIAL NERVE BLOCK BY LOCAL ANAESTHETICS . . . . . 565

GRAY, E. G. AND GUILLERY, R. W. THE BASIS FOR SILVER STAINING OF SYNAPSES OF THE MAMMALIAN SPINAL CORD: A LIGHT AND ELECTRON MICROSCOPE STUDY. . . . . 581

FOX, R. H., GOLDSMITH, R., KIDD, D. J. AND LEWIS, G. P. BRADYKININ AS A VASODILATOR IN MAN . . . . . 589

HATEMI, N. AND McCANCE, R. A. THE RESPONSE OF PIGLETS TO AMMONIUM CHLORIDE . . . . . 603

D'SILVA, J. L. AND LEWIS, A. F. THE MEASUREMENT OF BRONCHOCONSTRICTION *IN VIVO* . . . . . 611

*Printed in Great Britain at the University Press, Cambridge*
*(Brooke Crutchley, University Printer)*